US009080185B2

(12) **United States Patent**
Liao et al.

(10) **Patent No.: US 9,080,185 B2**
(45) **Date of Patent: Jul. 14, 2015**

(54) **RECOMBINANT FLAVIVIRAL CONSTRUCTS AND USES THEREOF**

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(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 181 days.

(21) Appl. No.: **13/825,049**

(22) PCT Filed: **Sep. 20, 2011**

(86) PCT No.: **PCT/US2011/052379**
§ 371 (c)(1), (2), (4) Date: **Jun. 10, 2013**

(87) PCT Pub. No.: **WO2012/040218**
PCT Pub. Date: **Mar. 29, 2012**

(65) **Prior Publication Data**

US 2013/0243809 A1 Sep. 19, 2013

Related U.S. Application Data

(60) Provisional application No. 61/384,720, filed on Sep. 21, 2010.

(51) **Int. Cl.**

C12N 15/86	(2006.01)
A61K 39/12	(2006.01)
A61K 39/00	(2006.01)

(52) **U.S. Cl.**
CPC ***C12N 15/86*** (2013.01); ***A61K 39/12*** (2013.01); *A61K 2039/5254* (2013.01); *A61K 2039/5256* (2013.01); *C07K 2319/40* (2013.01); *C07K 2319/50* (2013.01); *C12N 2770/24122* (2013.01); *C12N 2770/24134* (2013.01); *C12N 2770/24143* (2013.01); *C12N 2770/32322* (2013.01); *C12N 2770/32334* (2013.01)

(58) **Field of Classification Search**
None
See application file for complete search history.

(56) **References Cited**

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(57) **ABSTRACT**

A recombinant viral construct for expressing an exogenous polypeptide in a cell and uses thereof are provided. The recombinant viral constructs are derived from Japanese encephalitis virus (JEV). The recombinant viral constructs encodes a fusion protein, which includes an exogenous (i.e., non-JEV) polypeptide and a JEV non-structural protein 1 (JEV NS1) or a segment thereof. Particularly, the exogenous polypeptide is inserted into the carboxyl-terminus of the JEV NS1, and the production of the recombinant fusion protein does not affect viral replication. Upon infection a cell with such recombinant viral constructs, JEV particles comprising limited multiplicative virions (LMV) may be produced. Each LMV comprises the as-described JEV replicon. The JEV particles are useful in eliciting an immune response to the exogenous polypeptide in a host and thereby confer the host with protective immunization against the exogenous polypeptide.

20 Claims, 15 Drawing Sheets

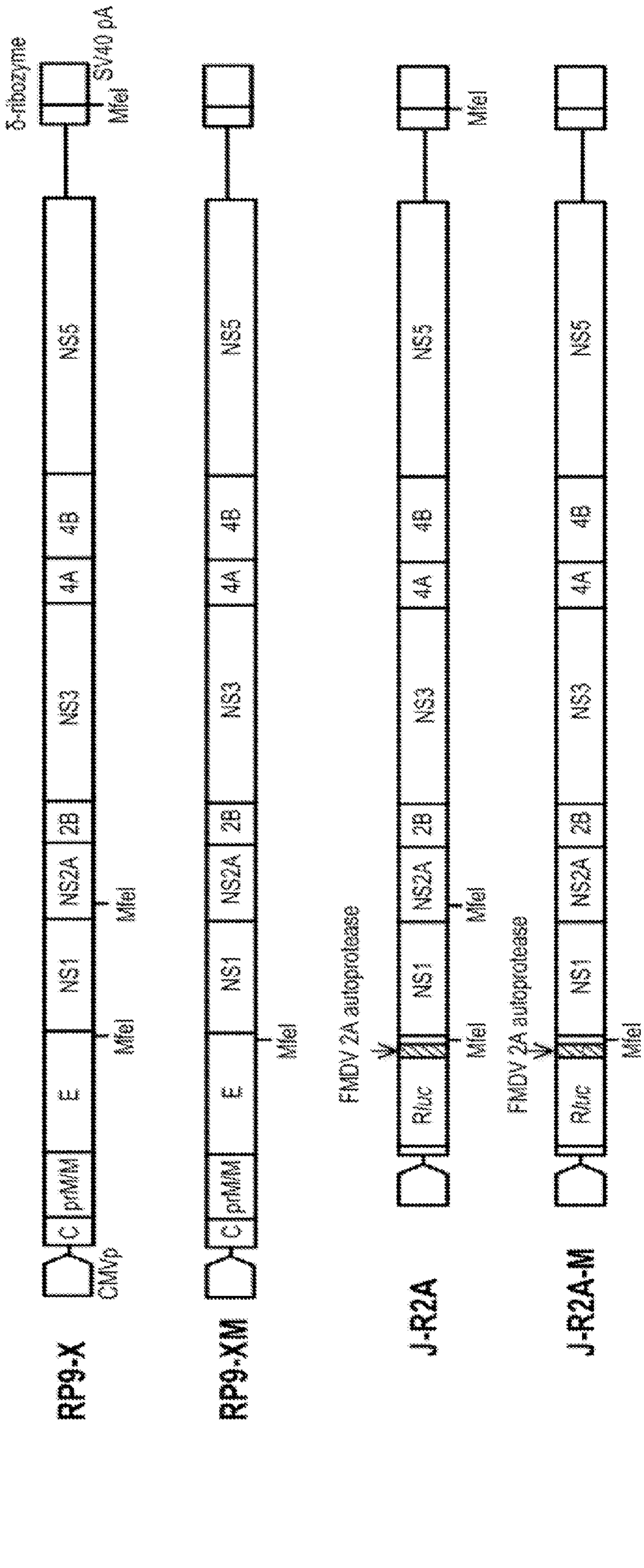

RP9-X
RP9-XM
J-R2A
J-R2A-M
CMVp
C
prM/M
E
NS1
NS2A
2B
NS3
4A
4B
NS5
MfeI
δ-ribozyme
SV40 pA
FMDV 2A autoprotease
Rluc

FIG 1

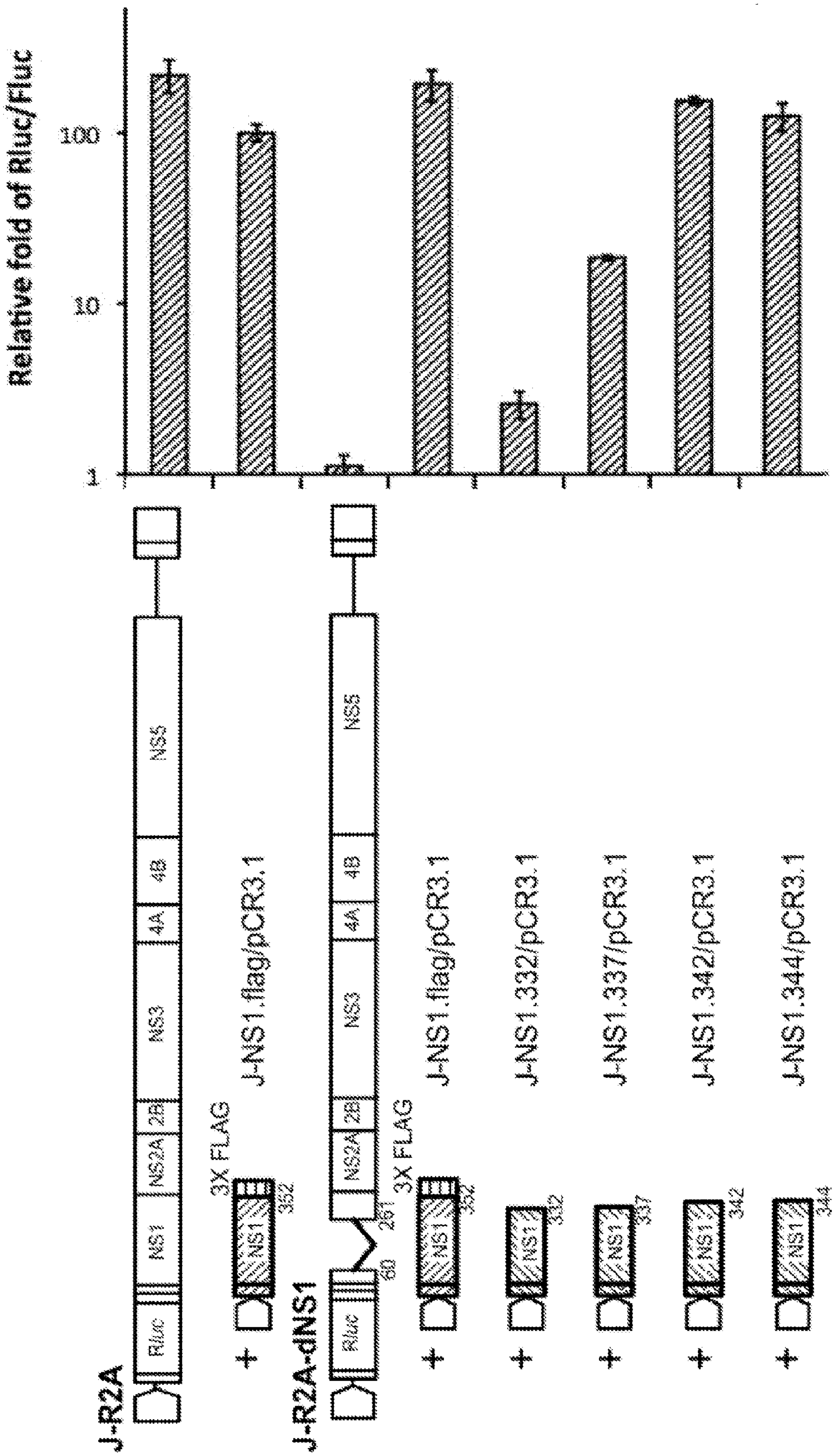
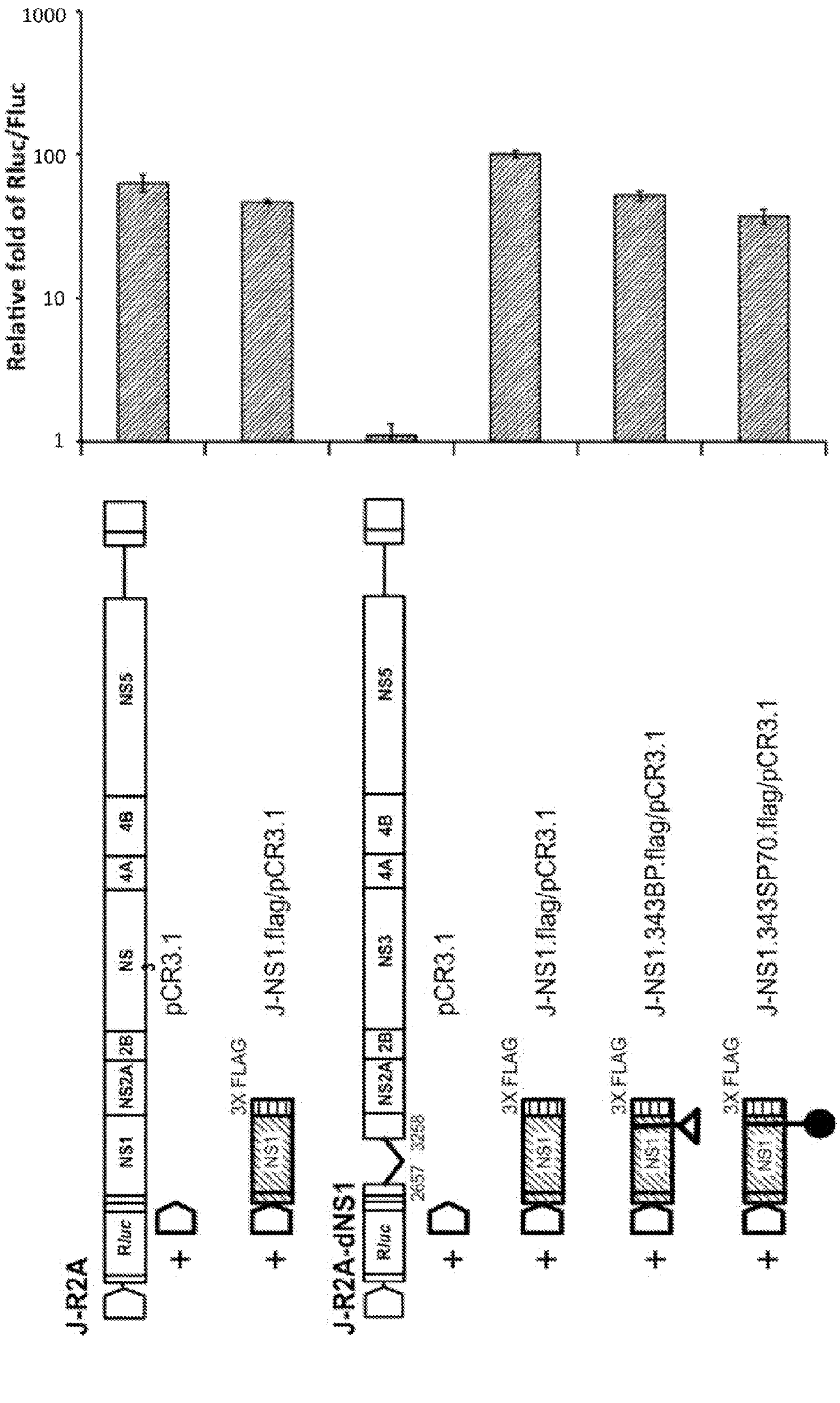

Relative fold of Rluc/Fluc
1
10
100
J-R2A
Rluc
NS1
NS2A
2B
NS3
4A
4B
NS5
3X FLAG
352
J-NS1.flag/pCR3.1
J-R2A-dNS1
60
251
J-NS1.332/pCR3.1
332
J-NS1.337/pCR3.1
337
J-NS1.342/pCR3.1
342
J-NS1.344/pCR3.1
344
+

FIG 2



Relative fold of Rluc/Fluc
1000
100
10
1
J-R2A
Rluc
NS1
NS2A
2B
NS
4A
4B
NS5
pCR3.1
3X FLAG
NS1
J-NS1.flag/pCR3.1
J-R2A-dNS1
2657
3258
NS3
pCR3.1
J-NS1.flag/pCR3.1
J-NS1.343BP.flag/pCR3.1
J-NS1.343SP70.flag/pCR3.1

FIG 3

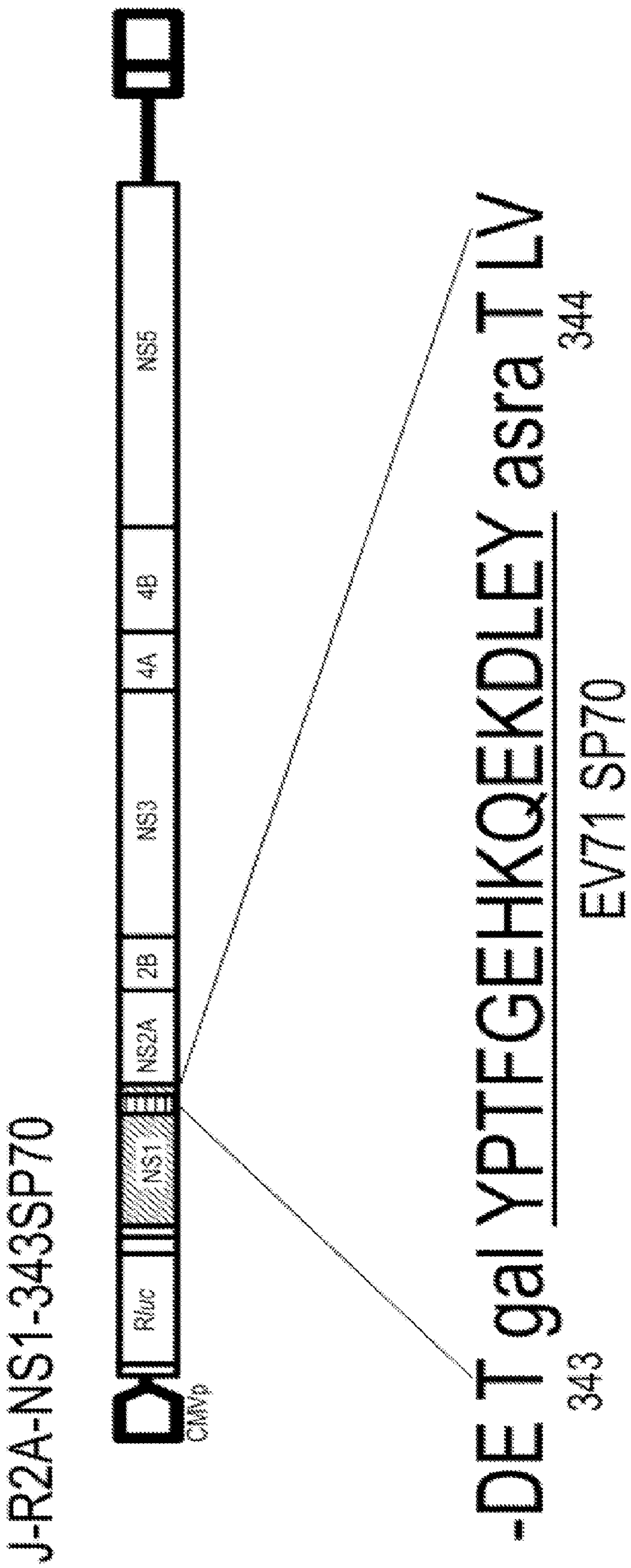

J-R2A-NS1-343SP70
CMVp
Rluc
NS1
NS2A
2B
NS3
4A
4B
NS5
-DE T gal YPTFGEHKQEKDLEY asra T LV
343
344
EV71 SP70

FIG 4

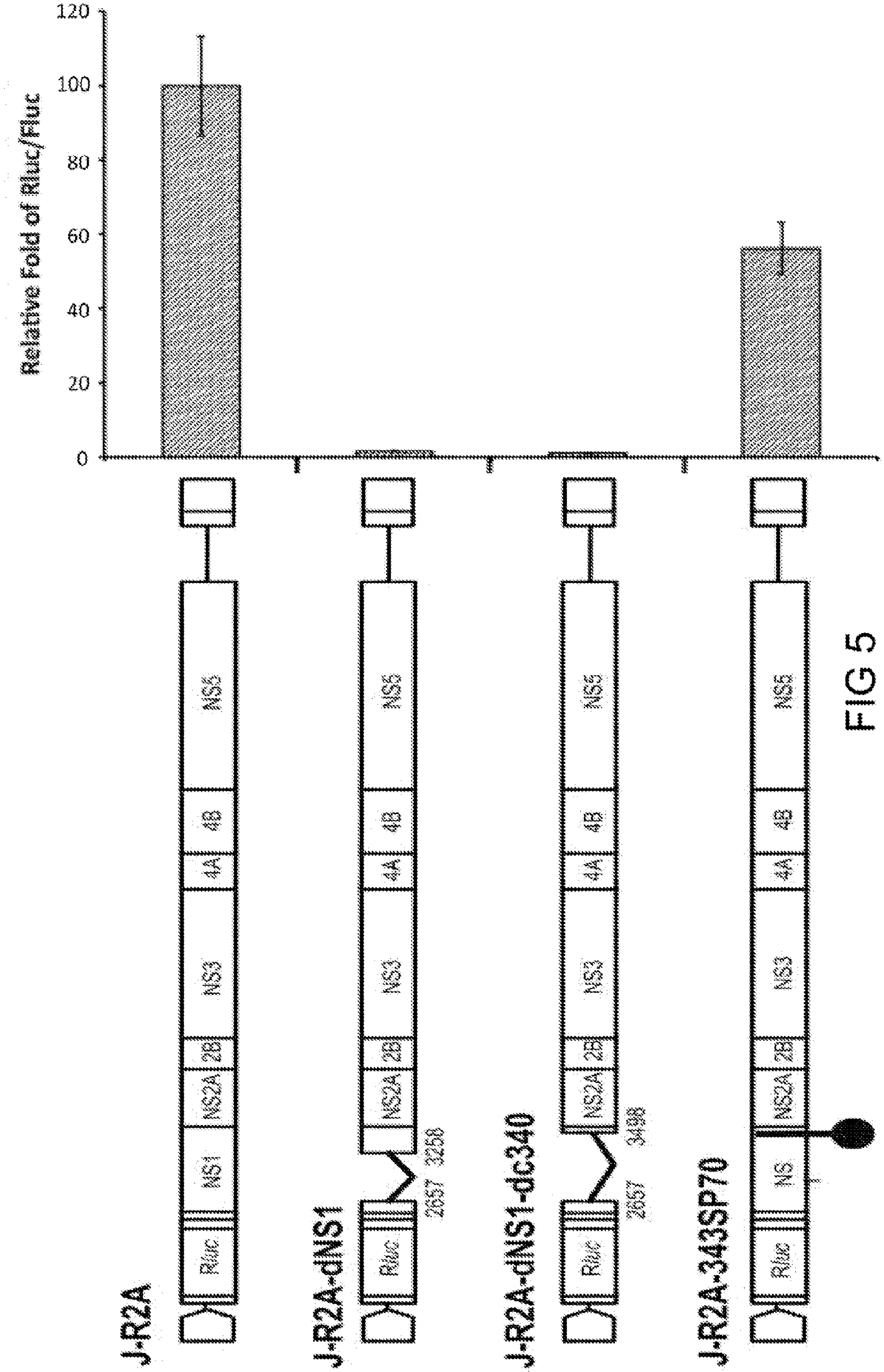

Relative Fold of Rluc/Fluc
120
100
80
60
40
20
0
J-R2A
J-R2A-dNS1
J-R2A-dNS1-dc340
J-R2A-343SP70
Rluc
NS1
NS
NS2A
2B
NS3
4A
4B
NS5
2657
3258
3498

FIG 5

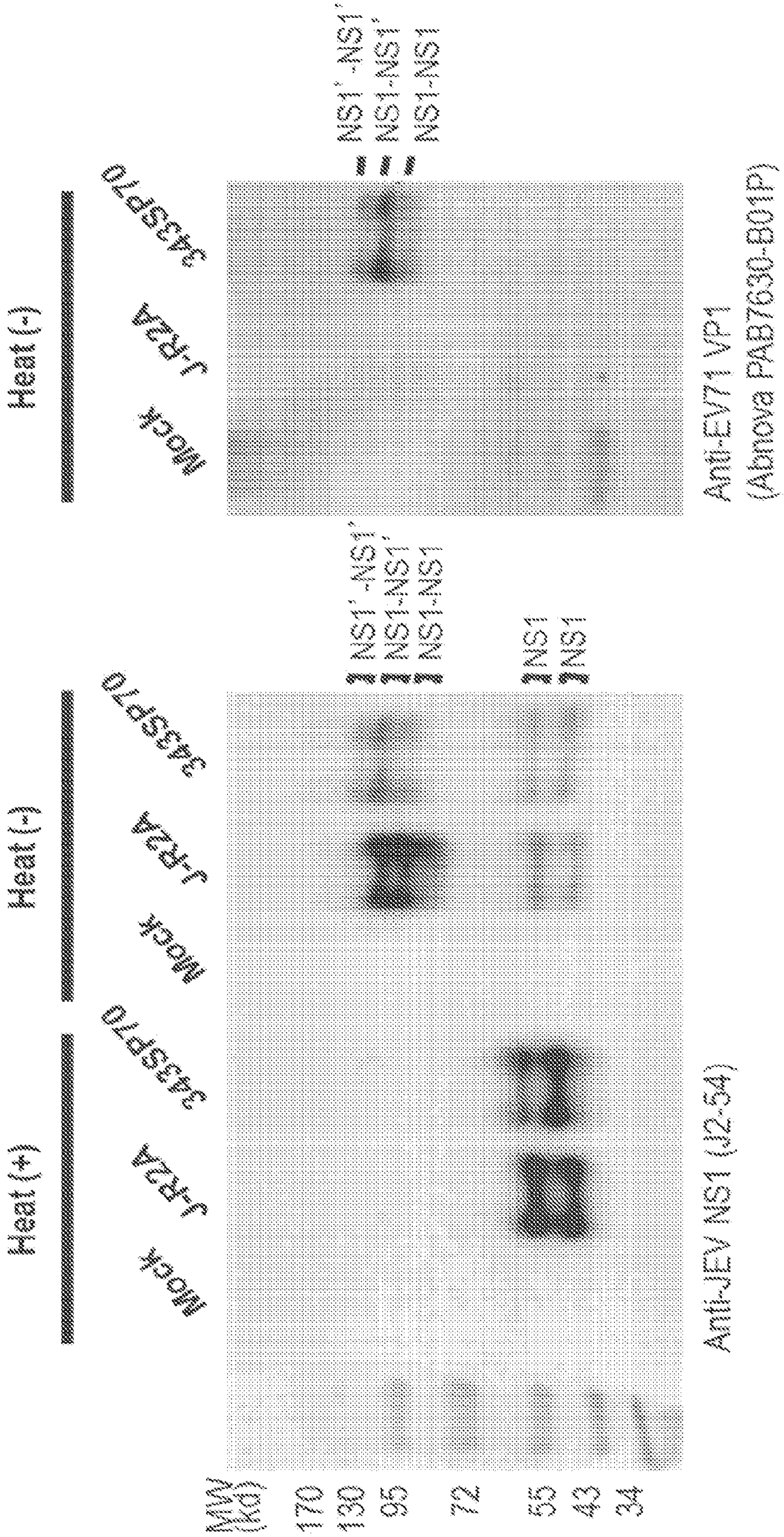

Heat (+)
Heat (-)
Heat (-)
Mock
J-R2A
34SP70
NS1'-NS1'
NS1-NS1'
NS1-NS1
NS1'
NS1
MW (Kd)
170
130
95
72
55
43
34
Anti-JEV NS1 (J2-54)
Anti-EV71 VP1
(Abnova PAB7630-B01P)

FIG 6

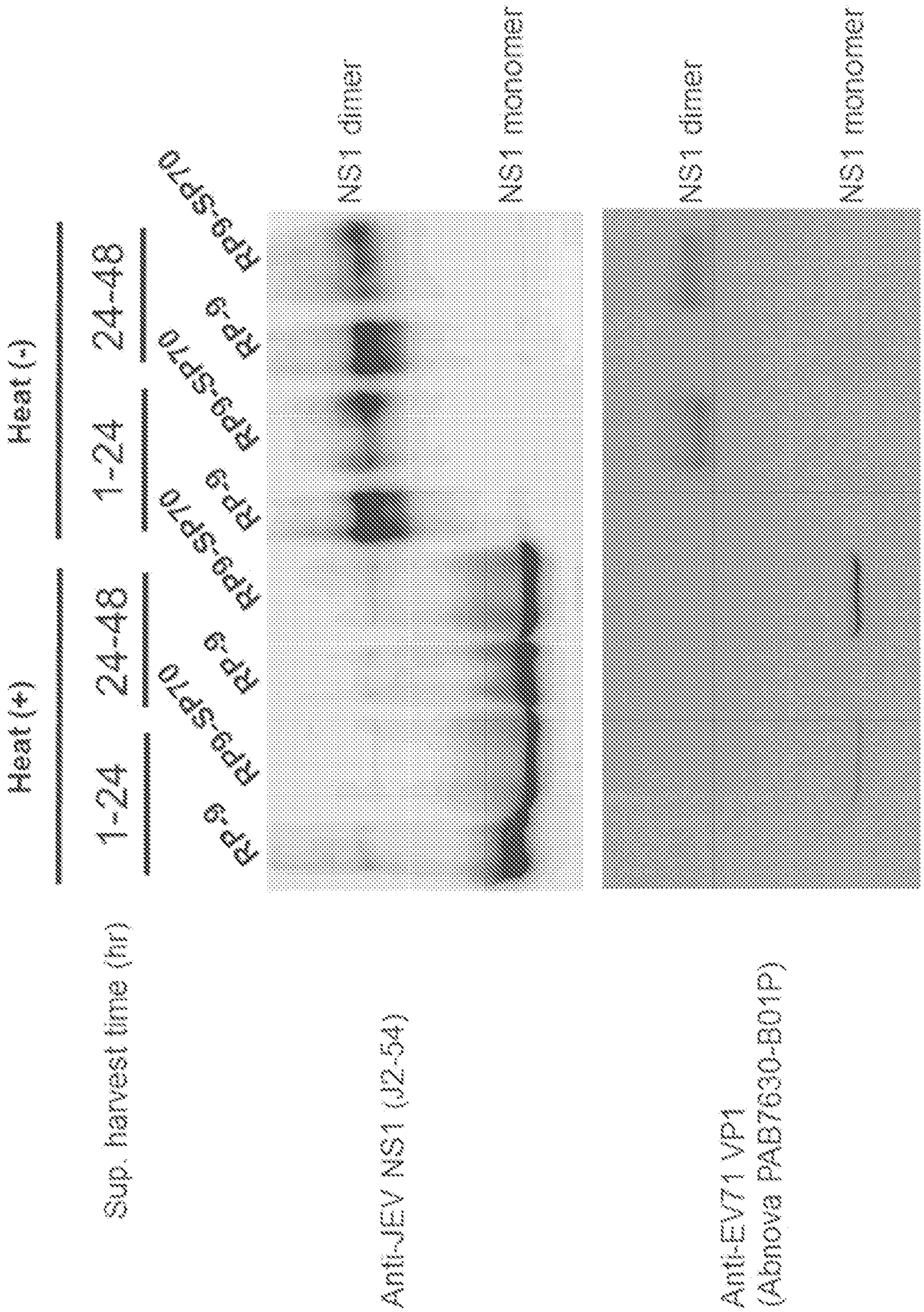

Sup. harvest time (hr)
Heat (+)
Heat (-)
1-24
24-48
1-24
24-48
RP9
RP9-SP70
RP9
RP9-SP70
RP9
RP9-SP70
RP9
RP9-SP70
NS1 dimer
NS1 monomer
Anti-JEV NS1 (J2-54)
NS1 dimer
NS1 monomer
Anti-EV71 VP1
(Abnova PAB7630-B01P)

FIG 7

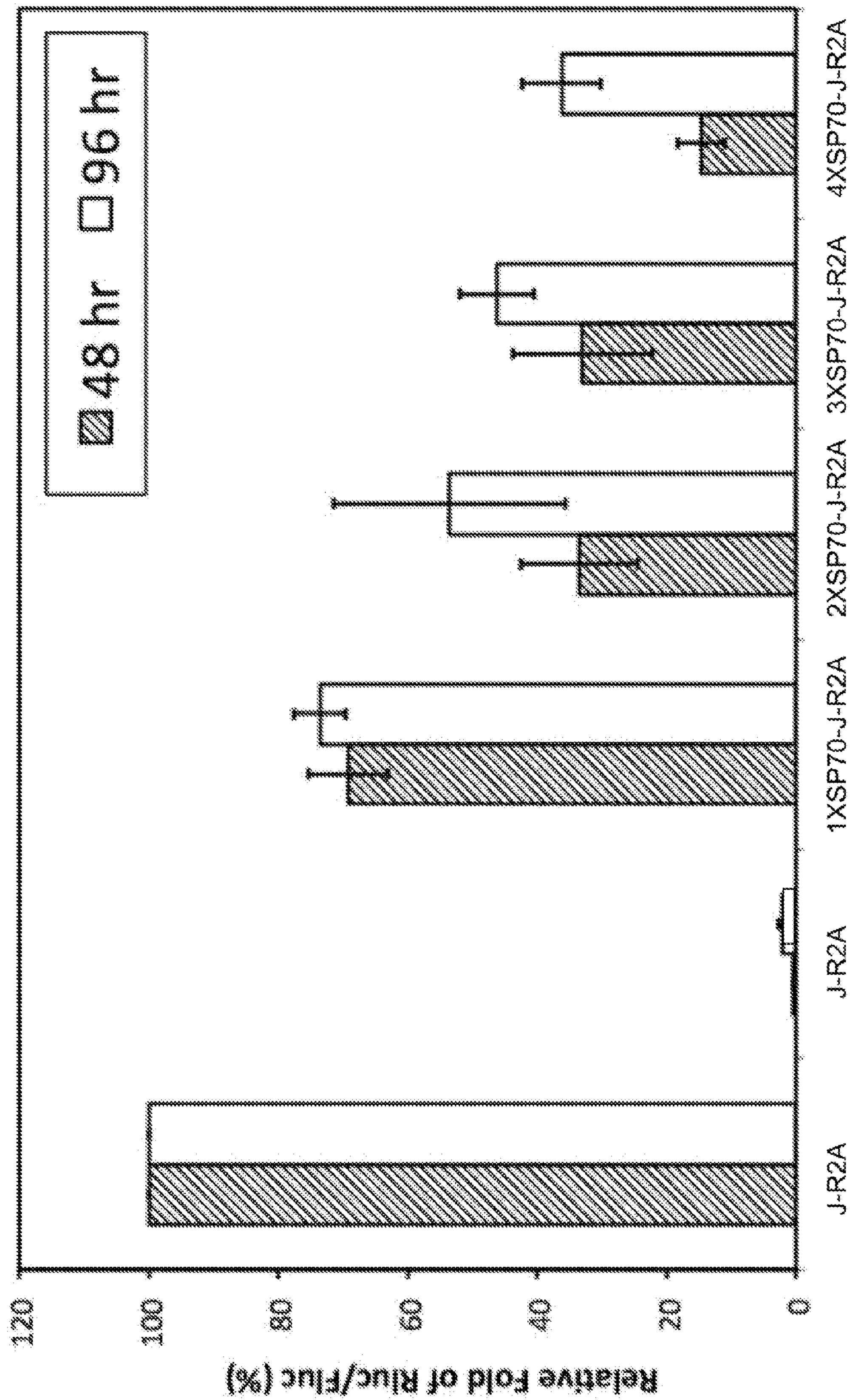

48 hr
96 hr
Relative Fold of Rluc/Fluc (%)
120
100
80
60
40
20
0
J-R2A
J-R2A
1XSP70-J-R2A
2XSP70-J-R2A
3XSP70-J-R2A
4XSP70-J-R2A

FIG 8

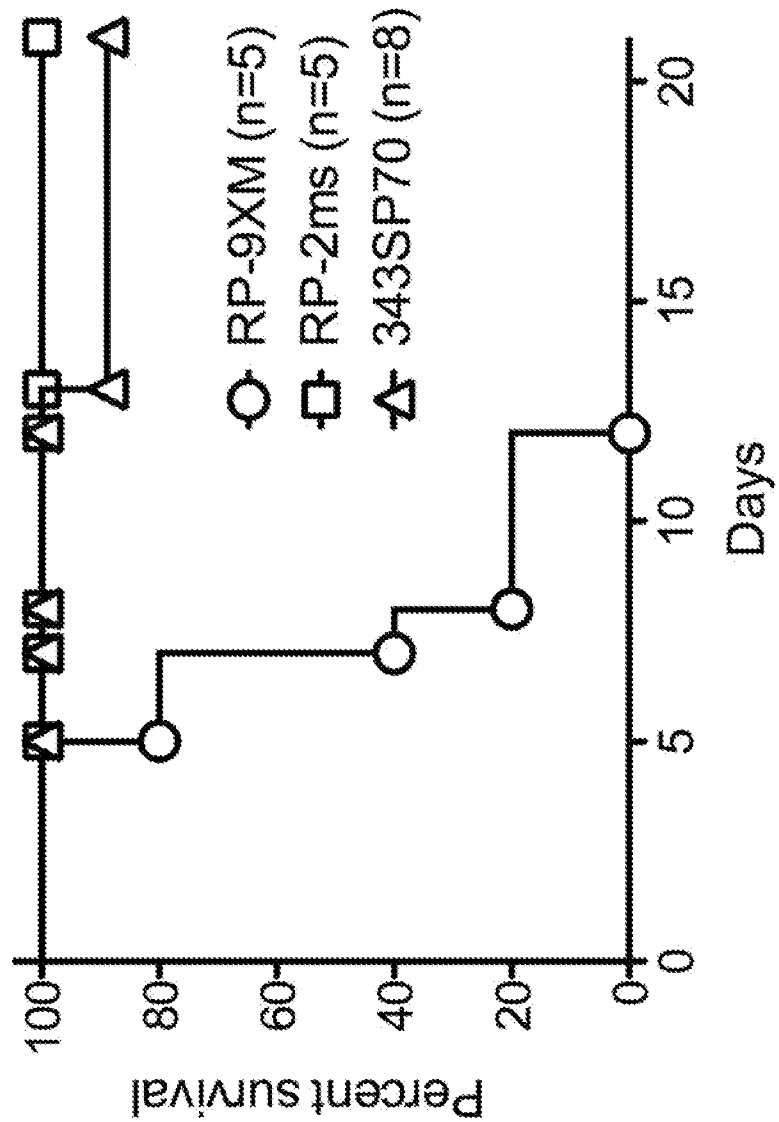
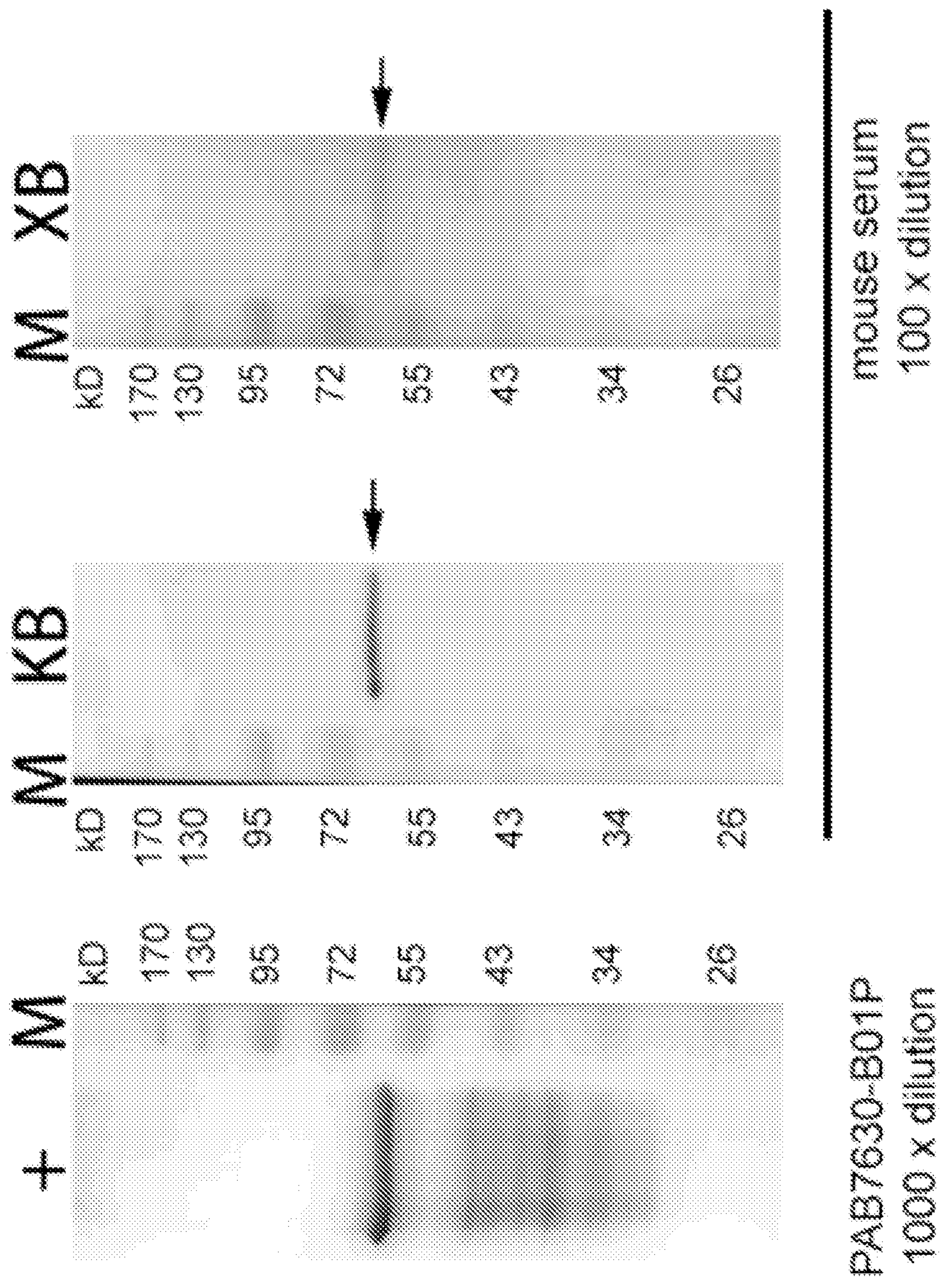

Percent survival
100
80
60
40
20
0
0
5
10
15
20
Days
RP-9XM (n=5)
RP-2ms (n=5)
343SP70 (n=8)

FIG 9



M
XB
kD
170
130
95
72
55
43
34
26
mouse serum
100 x dilution
M
KB
kD
170
130
95
72
55
43
34
26
+
M
kD
170
130
95
72
55
43
34
26
PAB7630-B01P
1000 x dilution

FIG 10

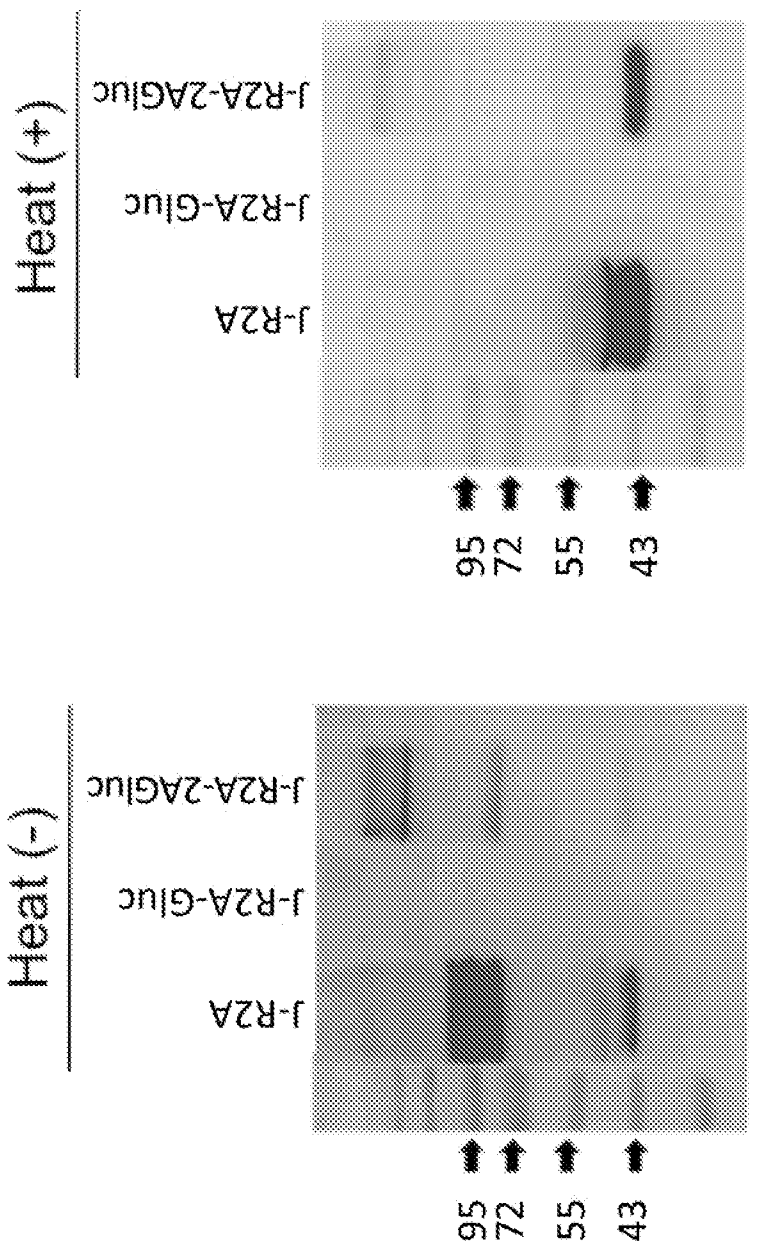

Heat (+)
J-R2A-2AGluc
J-R2A-Gluc
J-R2A
95
72
55
43
Heat (-)
J-R2A-2AGluc
J-R2A-Gluc
J-R2A
95
72
55
43

FIG 11

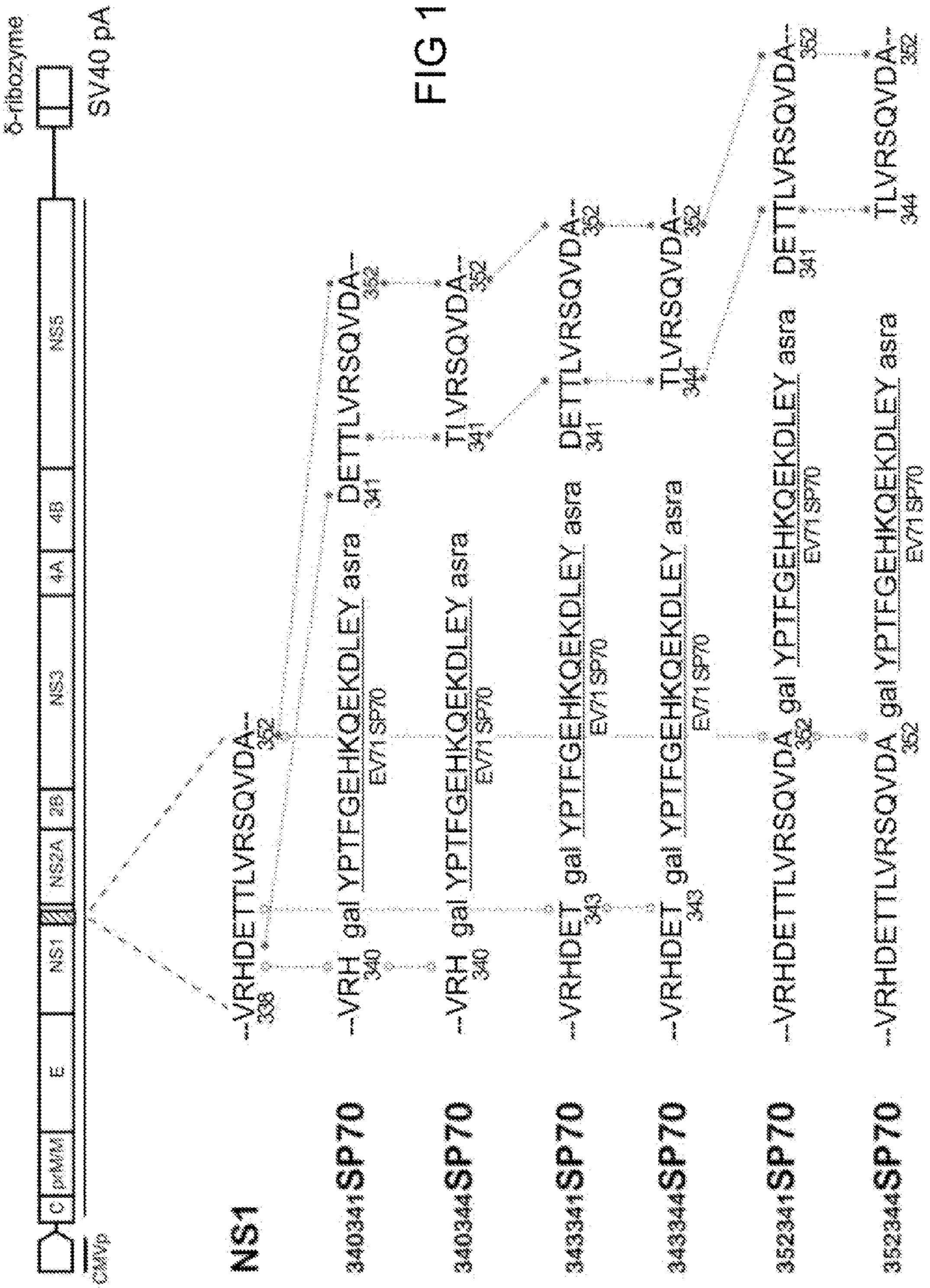

δ-ribozyme
SV40 pA
CMVp
C
prM/M
E
NS1
NS2A
2B
NS3
4A
4B
NS5
NS1
--VRHDETTLVRSQVDA--
338
352
$_{340341}$SP70
--VRH gal YPTFGEHKQEKDLEY asra DETTLVRSQVDA--
340
EV71 SP70
341
352
$_{340344}$SP70
--VRH gal YPTFGEHKQEKDLEY asra TLVRSQVDA--
340
EV71 SP70
344
352
$_{343341}$SP70
--VRHDET gal YPTFGEHKQEKDLEY asra DETTLVRSQVDA--
343
EV71 SP70
341
352
$_{343344}$SP70
--VRHDET gal YPTFGEHKQEKDLEY asra TLVRSQVDA--
343
EV71 SP70
344
352
$_{352341}$SP70
--VRHDETTLVRSQVDA gal YPTFGEHKQEKDLEY asra DETTLVRSQVDA--
352
EV71 SP70
341
352
$_{352344}$SP70
--VRHDETTLVRSQVDA gal YPTFGEHKQEKDLEY asra TLVRSQVDA--
352
EV71 SP70
344
352

FIG 12

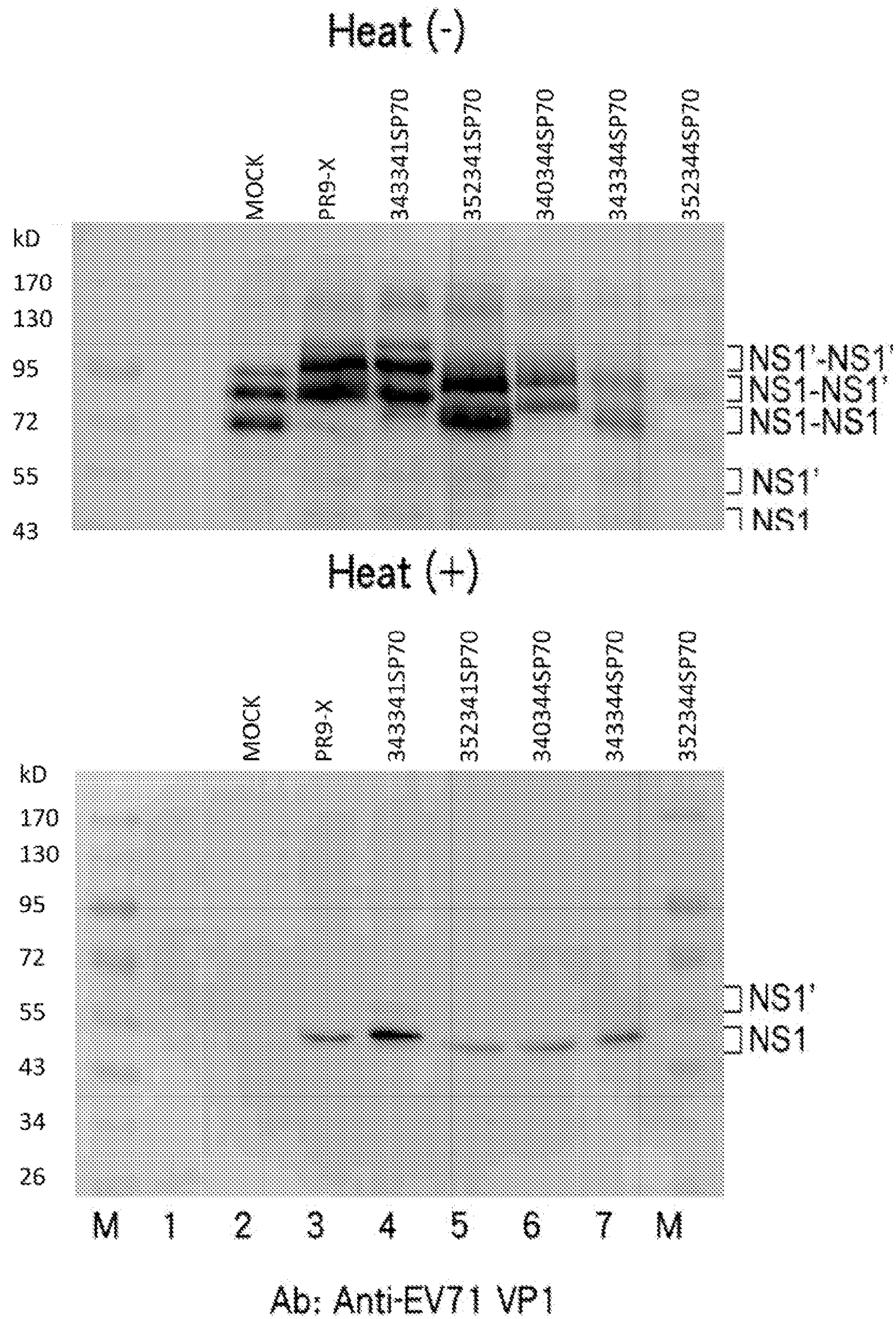

Heat (-)
MOCK
PR9-X
343341SP70
352341SP70
340344SP70
343344SP70
352344SP70
kD
170
130
95
72
55
43
NS1'-NS1'
NS1-NS1'
NS1-NS1
NS1'
NS1
Heat (+)
MOCK
PR9-X
343341SP70
352341SP70
340344SP70
343344SP70
352344SP70
kD
170
130
95
72
55
43
34
26
NS1'
NS1
M 1 2 3 4 5 6 7 M
Ab: Anti-EV71 VP1

FIG 13

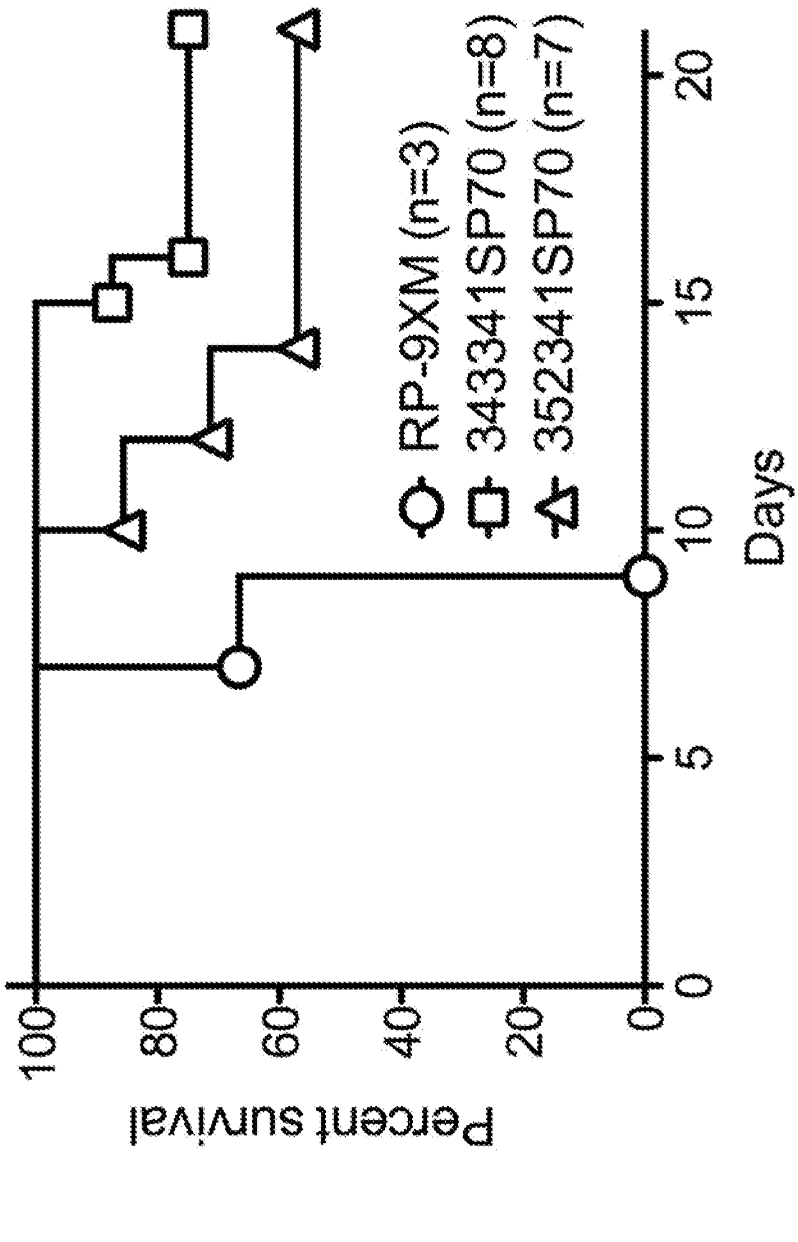

Percent survival
100
80
60
40
20
0
0
5
10
15
20
Days
RP-9XM (n=3)
343341SP70 (n=8)
352341SP70 (n=7)

FIG 14

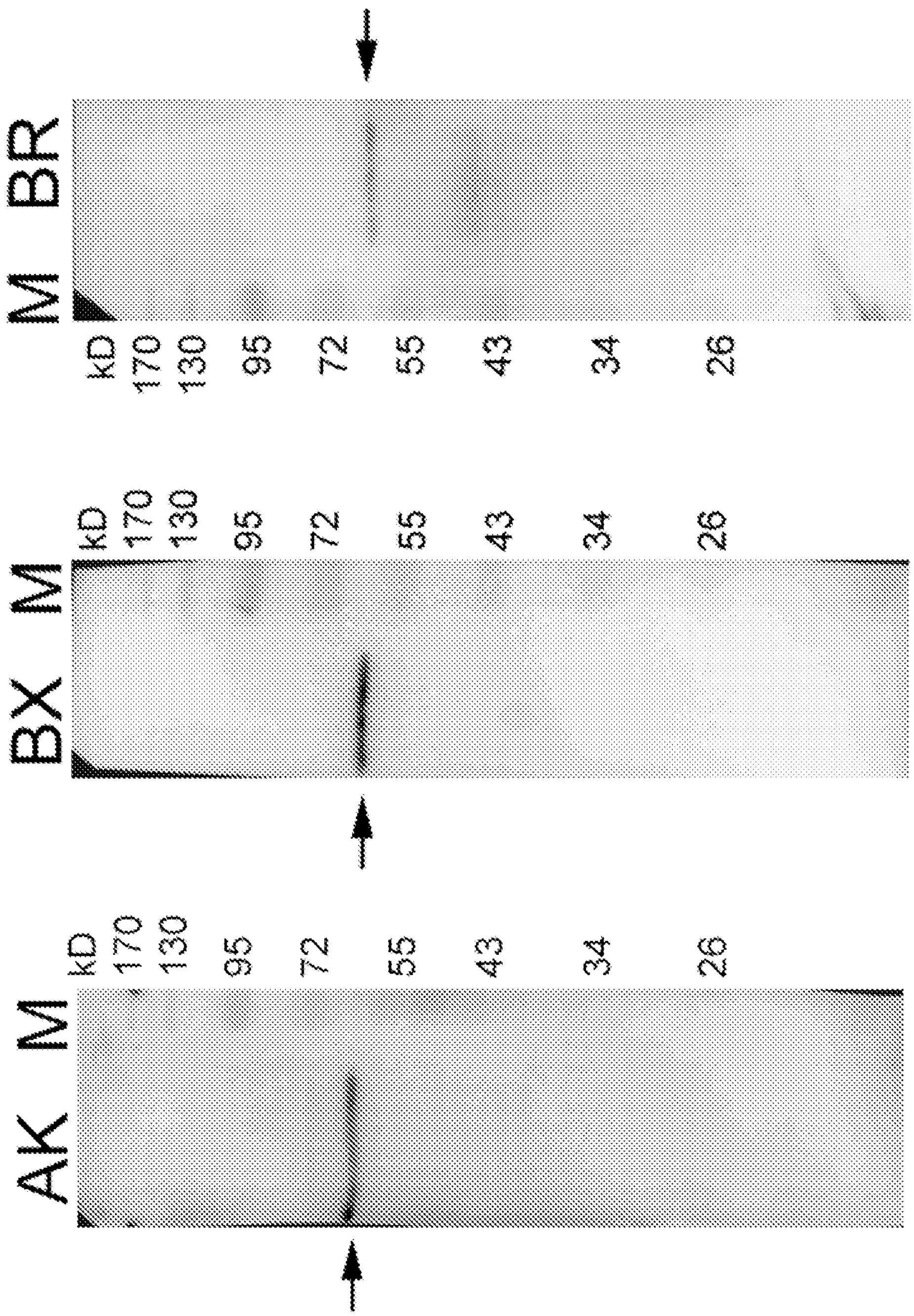

M BR
kD 170 130 95 72 55 43 34 26
BX M
kD 170 130 95 72 55 43 34 26
AK M
kD 170 130 95 72 55 43 34 26

FIG 15

RECOMBINANT FLAVIVIRAL CONSTRUCTS AND USES THEREOF

CROSS-REFERENCE TO RELATED APPLICATION

This application is the U.S. national phase of International Application No. PCT/US2011/052379, filed Sep. 20, 2011, which designated U.S. and claims priority to U.S. Provisional Application No. 61/384,720, filed Sep. 21, 2010, the entirety of which is incorporated herein by reference.

FIELD OF THE INVENTION

The present disclosure relates generally to the field of recombinant viruses and induction of specific immunity in a host. More particularly, the disclosure relates to the use of a recombinant virus vector for presenting an exogenous polypeptide antigen to a host and thereby elicits an immune response in the host to the exogenous polypeptide antigen.

BACKGROUND OF THE INVENTION

Replicon-based expression vectors have been developed for various RNA virus families, including alphaviruses, picornaviruses and flaviviruses. For example, expression system utilizing Yellow Fever viruses (YFV) for the presentation of foreign proteins or polypeptides that act as immunogens or therapeutic agents in host cells are well known. However, expression of foreign proteins or polypeptides in the prior art expression systems or vectors is limited to the structural regions of the flaviviruses.

The present invention overcomes the disadvantages and defects of the prior expression systems or vectors in that it provides the ability for foreign proteins or polypeptides to be functionally expressed in the nonstructural region of the virus, particularly, the C-terminus of a secreted non-structure protein 1 of a flavivirus.

SUMMARY

The present invention provides recombinant viral constructs derived from flaviviruses such as Japanese encephalitis virus (JEV) and dengue viruses (DEN) and uses thereof. The recombinant viral construct encodes a fusion protein, which comprises an exogenous (i.e., non-JEV or non-DEN) polypeptide antigen and a flaviviral non-structural protein 1 (NS1) or a segment thereof. Particularly, the exogenous polypeptide antigen is inserted at the carboxyl-terminus of the flavivirus NS1, such as JEV NS1, and the production of the recombinant fusion protein does not affect viral replication. Upon infection a cell with such recombinant viral constructs, flavivirus particles comprising limited multiplicative virions (LMV) may be produced. Each LMV comprises the flaviviral replicon or genome described above. The flavivirus particles are useful in eliciting an immune response to the exogenous polypeptide in a host and thereby confer the host with protective immunization against the exogenous polypeptide.

It is therefore a first aspect of the present invention to provide an isolated recombinant viral construct for expressing an exogenous polypeptide in a cell. The isolated recombinant viral construct comprises a flaviviral replicon comprising a nucleic acid encoding a fusion protein comprising a non-structural protein 1 (NS1) or a segment thereof and the exogenous polypeptide, wherein the exogenous polypeptide is at least 6 amino acids in length and is inserted into the

carboxyl-terminus of the NS1, and the production of the fusion protein does not affect viral replication.

The flaviviral replicon is a Japanese encephalitis virus (JEV) replicon or a dengue viruses (DEN) replicon. Preferably, the flaviviral replicon is JEV replicon.

In the case where the JEV replicon is employed, the JEV replicon comprises a nucleic acid encoding a fusion protein comprising, in sequence, a JEV non-structural protein 1 (JEV NS1) segment, the exogenous polypeptide, and a tail polypeptide. The JEV NS1 segment comprises at least amino acid residues 1 to 340 of the JEV NS1; the exogenous polypeptide has at least 6 amino acids; and the tail polypeptide comprises at least amino acid residues 344 to 352 of the JEV NS1.

According to some embodiments, the exogenous polypeptide comprises an immunogenic segment, such as an Enterovirus 71 (EV71) SP70 antigen, an EV71 VP1 antigen, a hepatitis B virus surface antigen or an immunogenic portion thereof. According to one embodiment, the exogenous polypeptide is at least 6 amino acids in length; and preferably at least 15 amino acids in length.

Optionally, the exogenous polypeptide may further comprise a protease segment preceding the immunogenic segment. The protease segment may comprises a Foot-and-Mouth Disease virus 2A (FMDV-2A) peptide to efficiently separate the exogenous polypeptide from the engineered NS1 fusion protein. In this embodiment, the recombinant viral construct may have a higher insertion capacity; for example, the exogenous polypeptide may be at least 50 amino acids in length; preferably at least 100 amino acids in length; and more preferably, at least 150 amino acids in length.

The flaviviral replicon comprises a promoter operable linked thereto, so that the fusion protein containing the exogenous polypeptide and the flavivirus NS1 may be expressed and subsequently secreted out of the cell. The cell may be any of a baby hamster kidney cells (e.g., BHK-21 cell), an *Aedes albopictus* C6/36 mosquito cell line (C6/36 cell), or Vero cell. Preferably, the cell is the BHK-21 cell.

The isolated recombinant viral construct may further comprise nucleic acids encoding flavivirus structural proteins (e.g., structural proteins C, prM, E and etc) required for packaging the flaviviral replicon into a flavivirus particle. Preferably, the nucleic acids encoding flavivirus structural proteins are engineered into the flavivirus genome.

It is therefore a second aspect of this invention to provide an isolated recombinant flavivirus particle, which comprises a virion unit comprising the recombinant viral construct of this invention.

It is a third aspect of this invention to provide a method of eliciting an immune response in a host. The method comprises steps of administering the isolated recombinant flavivirus particle of this invention to the host, wherein said administering provides for expression of the exogenous polypeptide that results in induction of an immune response in the host to the exogenous polypeptide. Preferably, the host is a cell or a mammal. More preferably, the mammal is a human.

In a forth aspect, the present invention relates to a cell transfected with a recombinant viral construct in accordance with the first aspect and embodiments of the present invention.

Further, in a fifth aspect of the present invention there is provided a kit for obtaining a transfected cell in accordance with the forth aspect and embodiments of the present invention. The kit comprises an isolated recombinant nucleic acid construct of the present invention and instructions for the use of said isolated recombinant construct.

BRIEF DESCRIPTION OF THE DRAWINGS

The present description will be better understood from the following detailed description read in light of the accompanying drawings, wherein:

FIG. **1** is a schematic representation of the recombinant constructs of RP9-X, RP9-XM, J-R2A and J-R2A-M in accordance with one embodiment of this invention;

FIG. **2** depicts the effects of deletion in the C-terminus region of NS1 on viral replication ability analyzed by trans-complementation assays in accordance with one embodiment of this invention;

FIG. **3** depicts the replication ability of the recombinant JEV virus, which comprises an Enterovirus 71 (EV71) SP70 antigen inserted between residues 343 and 344 of JEV NS1, analyzed by trans-complementation assays in accordance with one embodiment of this invention;

FIG. **4** is a schematic representation of the recombinant JEV construct (i.e., J-R2A-NS1-343SP70) having an Enterovirus 71 (EV71) SP70 antigen with linker (galYPTFGEHKQEKDLEYasra, SEQ ID NO: 11) inserted between residues 343 and 344 of JEV NS1 (SEQ ID NO: 16) in accordance with one embodiment of this invention;

FIG. **5** depicts the replication ability of defective replicons J-R2A-dNS1 including J-R2A-dNS1, J-R2A-dNS1-dc340 and J-R2A-NS1-343SP70 analyzed by trans-complementation assays in accordance with one embodiment of this invention;

FIG. **6** depicts the expression of the fusion protein (i.e., 343SP70) by the recombinant JEV virus in BHK-21 cells analyzed by western blot in accordance with one embodiment of this invention;

FIG. **7** depicts the secretion ability of the expressed fusion protein (i.e., 343SP70) analyzed by western blot in accordance with one embodiment of this invention;

FIG. **8** depicts the replication ability of the recombinant replicons capable of expressing fusion proteins comprising 1, 2, 3 and 4 copies of EV71 SP70 epitopes, respectively; and analyzed by trans-complementation assays in accordance with one embodiment of this invention;

FIG. **9** depicts survival plotted as the percentage of survival animals versus time following injection of RP-9XM, RP-2 ms and 343SP70 (i.e., the recombinant JEV virus of this invention), respectively, in accordance with one embodiment of this invention;

FIG. **10** depicts the responsiveness of mice immunized with 343SP70 to P3386 antigen analyzed by western blot in accordance with one embodiment of this invention;

FIG. **11** depicts the secretion ability of the expressed fusion protein (i.e., JR2A-Glu and JR2A-2A-Glu) analyzed by western blot in accordance with one embodiment of this invention;

FIG. **12** summarizes various insertion positions for the EV71 SP70 antigen with linker (galYPTFGEHKQEKDLEYasra, SEQ ID NO: 11) inserted between various amino acid residues of JEV NS1 (SEQ ID NO. 16) according to embodiments of this invention (the fragment labeled NS1 illustrates a.a. 338-352 of SEQ ID NO: 16; $_{340341}$SP70 illustrates SEQ ID NO: 11 inserted between a.a. 340 and 341 of SEQ ID NO: 16; for $_{340344}$SP70 the insertion is between a.a. 340 and 344 with deletion of a.a. 341-343); for $_{343341}$SP70 the insertion is between a.a. 343 and 341 (with duplication of a.a. 341-343 after the insert); for $_{343344}$SP70 the insertion is between a.a. 343 and 344; for 352341SP70 the insertion is between a.a. 352 and a.a. 341 (with a duplication of a.a. 341-352); and for $_{352344}$SP70 the insertion is between a.a. 352 and a.a. 344 (with a duplication of a.a. 344-352);

FIG. **13** depicts the secretion ability of the fusion proteins expressed by recombinant viral constructs of FIG. **12**, which is analyzed by western blot in accordance with one embodiment of this invention;

FIG. **14** depicts survival plotted as the percentage of survival animals versus time following injection of RP-9XM and recombinant JEV viruses of this invention (i.e., $_{343341}$SP70 and $_{352341}$SP70), respectively in accordance with one embodiment of this invention; and

FIG. **15** depicts the responsiveness of mice immunized with $_{352341}$SP70 to P3386 antigen analyzed by western blot in accordance with one embodiment of this invention.

DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

The detailed description provided below in connection with the appended drawings is intended as a description of the present examples and is not intended to represent the only forms in which the present example may be constructed or utilized. The description sets forth the functions of the example and the sequence of steps for constructing and operating the example. However, the same or equivalent functions and sequences may be accomplished by different examples.

Throughout this specification, unless otherwise indicated, "comprise", "comprises" and "comprising" are used inclusively rather than exclusively, so that a stated integer or a group of integers may include one or more other non-stated integers or groups of integers.

Unless otherwise defined herein, scientific and technical terms used in connection with the present invention shall have the meanings that are commonly understood by those of ordinary skill in the art. Further, unless otherwise required by context, singular terms shall include pluralities and plural terms shall include the singular. Generally, nomenclatures utilized in connection with, and techniques of, cell and tissue culture, molecular biology, and protein and oligo- or polynucleotide chemistry and hybridization described herein are those well known and commonly used in the art. Standard techniques are used for recombinant DNA, and tissue culture and transformation (e.g., lipofection). Enzymatic reactions and purification techniques are performed according to manufacturer's specifications or as commonly accomplished in the art or as described herein. The forgoing techniques and procedures are generally performed according to conventional methods well known in the art and as described in various general and more specific references that cited and discussed throughout the present specification. Standard techniques are used for pharmaceutical preparation, formulation, and delivery, and treatment of patients.

As utilized in accordance with the present disclosure, the following terms, unless otherwise indicated, shall be understood to have the following meanings:

The term "nucleic acid" as used herein designates single- or double-stranded RNA, mRNA, and DNA including cDNA and genomic DNA.

The term "polypeptide" as used herein is a general term to refer to native protein, fragments or analogs of a polypeptide sequence. Hence, native protein, fragments, and analogs are species of the polypeptide genus.

The term "recombinant" in the context of polypeptide coding regions and polypeptide encoded by such coding regions refers to non-native products wherein the coding regions, and typically the expression thereof, have been manipulated in vitro by man to differ from their occurrence in nature. The polypeptides utilized in the methods of the present invention may be produced in a number of different recombinant systems known in the art, including but not limited to, prokaryotic or eukaryotic system. For expression in an appropriate expression system, the desired viral polypeptide coding regions are operably linked to an expression vector and introduced into a host cell to enable expression. The coding region with the appropriate regulatory regions will be provided in proper orientation and reading frame to allow for expression. Methods for gene construction are known in the art. See, in particular, Molecular Cloning, A Laboratory Manual, Sambrook et al., Cold Spring Harbor Laboratory, Second Edition, Cold Spring Harbor, N.Y. (1989) and the references cited therein.

The term "isolated" is used in reference to a molecule, the term means that the molecule has been removed from its native environment. For example, a polynucleotide or a polypeptide naturally present in a living animal is not "isolated", but the same polynucleotide or polypeptide separated from the coexisting materials of its natural state is "isolated." Further, recombinant DNA molecules contained in a vector are considered isolated for the purpose of the present invention. Isolated DNA molecules include in vivo or in vitro DNA molecules. Isolated nucleic acid molecules further include synthetically produced molecules. Additionally, vector molecules contained in recombinant host cells are also isolated.

As used herein, the term "construct or vector" refers to agent (e.g., a plasmid or virus) used to transmit genetic material to a host cell. A construct or a vector may be composed of either DNA or RNA.

As used herein, the term "exogenous or heterologous polynucleotide sequence" refers to a second nucleotide sequence present in a constructor or a vector of the invention. The term "exogenous or heterologous polypeptide sequence" refers to any amino acid sequence encoded by an exogenous polynucleotide sequence contained in a vector of the invention. Heterologous polynucleotide sequence may encode proteins or RNA molecules normally expressed in cell type in which they are present or molecules not normally expressed therein.

The term "flavivirus" and "flaviviral" refer to members of the family Flaviviridae within the genus *Flavivirus*, which contains 65 or more related viral species. Typically, flavivirus are small, enveloped RNA viruses with peplomers comprising a single glycoprotein E. Other structural proteins are designated C (core) and M (membrane-like). Flaviviruses infect a wide range of vertebrates, and many are transmitted by arhropods such as ticks and mosquitoes. Particularly, non-limiting examples of flavivirus are West Nile virus (WNV), Kunjin virus, Yellow Fever virus (YFV), Japanese Encephalitis virus (JEV), Dengue virus (DEN), Usutu virus, St Louis Encephalitis virus (SLE) and tick-born encephalitis virus (TBEV). Preferably, flavivirus suitable for use in this invention are JEV and DEN.

The term "recombinant viral construct" refers to assembly which is capable of directing the expression of a sequence(s) or gene(s) of interest. Such viral constructs are comprised of a 5' sequence which is capable of initiating transcription of a viral RNA (e.g., JEV RNA), as well as sequences which, when expressed, code for biologically active viral nonstructural proteins. The viral construct may also include sequences from one or more structural and/or nonstructural protein genes or portions thereof, exogenous nucleic acid molecules which are of a size sufficient to allow production of viable virus, a 5' promoter which is capable of initiating the synthesis of viral RNA in vitro from cDNA, an exogenous sequence to be expressed, as well as one or more restriction sites for insertion of heterologous sequences.

The term "replicon" refers to a viral DNA or RNA molecule that is capable of directing its own amplification or self-replication in vivo, within a target cell. To direct its own replication, the DNA or RNA molecule may: (1) encode one or more polymerase, replicase, or other proteins which may interact with viral or host cell-derived proteins, nucleic acids or ribonucleoproteins to catalyze DNA or RNA amplification; and (2) contain cis DNA or RNA sequences required for replication which may be bound by its self-encoded proteins, or non-self-encoded proteins, cell-derived proteins, nucleic acids or ribonucleoproteins, or complexes between any of these components. Flavivirus replicon RNA produced in cells as a result of its self-amplification either after RNA transfection or after plasmid DNA transfection, can be packaged into the secreted virus-like particles (VLPs) by providing structural proteins from a second vector.

The present invention, at least in part, arises from the unexpected finding that the C-terminus portion of non-structural protein 1 (NS-1) of a flavivirus (e.g., JEV) may tolerate exogenous polypeptide insertion, hence may serve as a good vehicle for presenting foreign epitope(s) to a host. Furthermore, the inclusion of the exogenous polypeptide within the flaviviral replicon does not significantly reduce replication capability of the flaviviral replicon.

It will be appreciated that the present invention contemplates a recombinant viral construct capable of presenting an immunogenic epitope of a virulent and/or pathogenic virus to a host to protectively immunize the host against said virulent and/or pathogenic virus. The invention may therefore be useful in human and veterinary medicine.

Therefore, in a preferred embodiment, the invention provides an isolated recombinant viral construct for expressing an exogenous polypeptide in a cell. The isolated recombinant viral construct comprises a JEV replicon comprising a nucleic acid encoding a fusion protein that comprises a JEV non-structural protein 1 (JEV NS1) or a segment thereof and an exogenous polypeptide, wherein the exogenous polypeptide is at least 6 amino acids in length and is inserted into the carboxyl-terminus of the JEV NS1, wherein the production of the exogenous polypeptide does not affect viral replication.

Specifically, the fusion protein comprises, in sequence, a JEV non-structural protein 1 (JEV NS1) segment, the exogenous polypeptide, and a tail polypeptide. Generally, the exogenous polypeptide has at least 6 amino acids, preferably, at least 15 amino acids; more preferable, at least 50 amino acids. In particular, the exogenous polypeptide to be inserted therein could have at least 6, 10, 15, 20, 30, 40, 50, 60, 70, 80, 90, or 100 amino acids. For example, some exogenous polypeptides described in the Examples hereinbelow each have a length of 22, 39, 56 and 73 amino acids.

The JEV NS1 segment comprises at least amino acid residues 1 to 340 of the JEV NS1, and the tail polypeptide comprises at least amino acid residues 344 to 352 of the JEV NS1. Throughout the present disclosure, a JEV NS1 segment comprising amino acid residues 1 to n of the JEV NS1 is denoted as JEV $NS1_n$, whereas a tail polypeptide comprising amino acid residues m to 352 of the JEV NS1 is denoted as tail $polypeptide_m$.

According to one embodiment of the present disclosure, the fusion protein comprises a JEV $NS1_{340}$, an exogenous polypeptide, and a tail $polypeptide_{341}$, such as the cDNA clone $_{340341}$SP70 described hereinbelow. In another embodiment, the fusion protein comprises a JEV $NS1_{343}$, an exogenous polypeptide, and a tail $polypeptide_{344}$, such as the cDNA clone $_{343344}$SP70 described hereinbelow. In still another embodiment, the fusion protein comprises a JEV

$NS1_{340}$, an exogenous polypeptide, and a tail polypeptide$_{344}$, such as the cDNA clone $_{340344}$SP70 described hereinbelow.

Without being bound by theory, it is believed that the presence of the consecutive amino acid residues 344 to 352 of the JEV NS1 at the end of the fusion protein is required for the expression and secretion of the fusion protein. Accordingly, in one embodiment, the fusion protein comprises a JEV $NS1_{352}$, an exogenous polypeptide, and a tail polypeptide$_{344}$, such as the cDNA clone $_{352344}$SP70 described hereinbelow.

In some cases, the presence of the consecutive amino acid residues 341 to 352 of the JEV NS1 at the end of the fusion protein is preferred. Therefore, the fusion protein comprises a JEV $NS1_{343}$, an exogenous polypeptide, and a tail polypeptide$_{341}$, such as the cDNA clone $_{343341}$SP70 described hereinbelow. In another embodiment, the fusion protein comprises a JEV $NS1_{352}$, an exogenous polypeptide, and a tail polypeptide$_{341}$, such as the cDNA clone $_{352341}$SP70 described hereinbelow.

According to the principles and spirits of the present disclosure, the exogenous polypeptide comprises an immunogenic segment, such as an EV71 SP70 antigen, an EV71 VP1 antigen, or an immunogenic portion thereof. According to one embodiment, the exogenous polypeptide is at least 6 amino acids in length; and preferably at least 15 amino acids in length. As a non-limiting example, the exogenous polypeptide is EV71 SP70 antigen having an amino acid sequence of YPTFGEHKQEKDLEY (SEQ ID NO: 10), which is 15 amino acids in length. In one embodiment, EV71 SP70 antigen sequence of galYPTFGEHKQEKDLEYasra (SEQ ID NO: 11), which is 22 amino acids in length including linker sequence in lowercase letter, is inserted between the JEV $NS1_{343}$ and the tail polypeptide$_{344}$. In other embodiments, immunogenic segments comprising 2, 3 or 4 copies of the above-described EV71 SP70 antigen sequence of YPTFGEHKQEKDLEY (SEQ ID NO: 10), which are 39, 56 and 73 amino acids in length, respectively, were inserted between the JEV $NS1_{343}$ and the tail polypeptide$_{344}$, with reduced viral replication or infection.

In optional embodiments, the exogenous polypeptide may further comprise a protease segment preceding the immunogenic segment thereby providing a recombinant viral construct that has a higher insertion capacity. The protease segment may comprise a Foot-and-Mouth Disease virus 2A (FMDV-2A) peptide. In this embodiment, the thus-obtained recombinant viral construct is capable of carrying an exogenous polypeptide having at least 100 amino acids in length. In particular, the exogenous polypeptide to be inserted therein could have a length of at least 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 210, 220, 230, 240, or 250 amino acids. For example, two exogenous polypeptides described in the Examples hereinbelow each have a length of 153 and 202 amino acids.

Still optionally, the exogenous polypeptide may further comprise a secretion-signal segment between the protease segment and the immunogenic segment, wherein the secretion-signal segment comprises a secretion signal peptide derived from *Gaussia* luciferase.

In one exemplary embodiment, the exogenous polypeptide has a total length of 153 amino acids that consists of an FMDV-2A peptide (NFDLLKLAGDVESNPGP, 17 amino acids), a secretion signal peptide derived from *Gaussia* luciferase (MGVKVLFALICIAVAEAGL, 19 amino acids), and amino acid 145th to 261st from EV71 VP1 (117 amino acids). The exogenous polypeptide was inserted between the JEV $NS1_{343}$ and the tail polypeptide$_{344}$. A suitable promoter is operably linked to the replicon so as to facilitate the amplification or self-replication of the viral replicon and the exogenous polypeptide linked thereto in the host cell. Suitable promoters include, but are not limited to, mammalian-operable promoters inducible through the bacterial lac operon (e.g., lac-regulated CMV or RSV promoter). A preferred promoter is a CMV promoter.

The host cell suitable for receiving the recombinant viral construct of this invention may be any of a BHK-21 cell, a C6/36 cell, a Vero cell, or other equivalent mammalian cells. Preferably, the host cell is a BHK-21 cell.

The present invention is characterized in that it provides the ability for exogenous proteins or polypeptides to be functionally expressed in the nonstructural region of the virus, particularly, nonstructural protein 1 (NS1) of a flavivirus. Nonstructural protein 1 contains two distinct domains or regions, and their precise roles in viral replication are not yet well understood. The C-terminus region is not conserved among known flaviviruses in length or in sequence, and multiple changes are tolerated. Insertion/deletion studies on the C-terminus region of JEV NS1 have been conducted in accordance with one example of this invention. The exogenous polypeptide having at least 6 amino acids may be inserted into the carboxyl-terminus of the NS1.

In one embodiment, Enterovirus 71 (EV71) SP70 antigen sequence of galYPTFGEHKQEKDLEYasra (SEQ ID NO: 11), which is 22 amino acids in length including linker sequence in lowercase letter, is inserted between the JEV $NS1_{343}$ and the tail polypeptide$_{344}$ (FIG. **4**). In other embodiments, immunogenic segments comprising 2, 3 or 4 copies of the above described Enterovirus 71 (EV71) SP70 antigen sequence of YPTFGEHKQEKDLEY (SEQ ID NO: 10), which are 39 (SEQ ID NO: 12), 56 (SEQ ID NO: 13) and 73 (SEQ ID NO: 14) amino acids in length, respectively were inserted between the JEV $NS1_{343}$ and the tail polypeptide$_{344}$, with reduced viral replication or infection (FIG. **8**). The isolated recombinant viral construct may further comprise nucleic acids encoding flavivirus structural proteins (e.g., structural proteins C, prM and E) required for packaging the flaviviral replicon of this invention into a flavivirus particle. Such nucleic acids are preferably engineered into the flaviviral genome.

Therefore, in a preferred embodiment, the invention provides an isolated recombinant flavivirus particle, comprising a virion unit comprising the isolated recombinant viral construct of this invention. The virion may be used as an immunogen (e.g., a live or killed vaccine) for eliciting a protective immune response to at least the exogenous polypeptide encoded by the flavivirus replicon of this invention in a subject. Accordingly, an immunotherapeutic composition or vaccine may be produced. The immunotherapeutic composition or vaccine may be used to prophylatically or therapeutically immunize animals such as human.

It is therefore a further aspect of this invention to provide a method of eliciting an immune response in a subject. The method comprises steps of administering the isolated recombinant flavivirus particle of this invention to the subject, wherein said administering provides for expression of the exogenous polypeptide that results in induction of an immune response in the subject to the exogenous polypeptide. The subject may be a vertebrate animal such as cows, sheep, dogs, cats, birds, pigs and etc. In other embodiment, the vertebrate animal is a mammal. More preferably, the mammal is a human.

In certain embodiments, it is contemplated that a transfected cell comprises a recombinant viral construct of the invention. The transfected cell is a cell type that can be used to express the exogenous polypeptide encoded by the construct. For examples, it is known that JEV vectors may be used to express exogenous polypeptides in mammalian cells, examples of which include, but are not limited to, BHK-21 cells and C6/36 cells; preferably, BHK-21 cells.

The present invention is also directed to a kit that supplies the elements necessary to conduct transfection of cells. The kit comprises an isolated recombinant viral construct of the invention and instructions for the use of said isolated recombinant construct.

In one embodiment, a kit for inserting a DNA sequence of interest into a recombinant viral construct is provided. The recombinant viral construct comprises a first nucleic acid sequence and a second nucleic acid sequence operably linked to the first nucleic acid sequence. The first nucleic acid sequence encodes a JEV NS1 segment comprising at least amino acid residues 1 to 340 of the JEV NS1, whereas the second nucleic acid sequence encodes a tail polypeptide comprising at least amino acid residues 344 to 352 of the JEV NS1. The DNA sequence of interest may be inserted between the first and second nucleic acid sequences in accordance with instructions provided with the kit to obtain the recombinant viral construct for expressing an exogenous polypeptide in a cell. In one optional embodiment, the kit may further comprise a third nucleic acid sequence positioned between the first and second nucleic acid sequences. The third nucleic acid sequence encodes an FMDV-2A peptide, and the DNA sequence of interest may be inserted between the third and second nucleic acid sequences in accordance with instructions provided with the kit to obtain the recombinant viral construct for expressing an exogenous polypeptide in a cell.

Alternatively, the DNA sequence of interest could be inserted into a recombinant viral construct in advanced. In this case, a kit comprising a recombinant viral construct for expressing an exogenous polypeptide in a cell is provided together with instructions for using the same. In one embodiment, the recombinant viral construct for expressing an exogenous polypeptide in a cell comprises, in sequence, the first nucleic acid sequence, the DNA sequence of interest, and the second nucleic acid sequence. In one optional embodiment, the recombinant viral construct for expressing an exogenous polypeptide in a cell comprises, in sequence, the first nucleic acid sequence, the third nucleic acid sequence, the DNA sequence of interest, and the second nucleic acid sequence.

In certain embodiments, any of the above mentioned kits may optionally comprise cells suitable to be infected by the recombinant viral construct.

Said instructions for the use of said isolated recombinant construct may include instructions as to the amount or concentration of the isolated recombinant viral construct provided. If the construct is provided dried, the instructions may teach how to reconstitute the construct into solution. The instructions may further teach how to introduce said construct into a cell. Additionally, the instructions can indicate various cell types that can be transfected with the construct and how to culture the transfected cells so that they will express a desired exogenous polypeptide. The instructions may also teach how to recover the desired exogenous polypeptide from a transfected cell or from a conditioned cell culture medium produced by a transfected cell. Instructions may be included in the kit in either printed or electronic form. Alternatively, the instructions can be provided by way of a link or internet address that provides access to instructions located on either an internet or extranet site. The internet site can be either publicly available or secure.

The individual components of the kits can be packaged in a variety of containers, e.g., vials, tubes, microtiter well plates, bottles, and the like. Other reagents can be included in separate containers and provided with the kit; e.g., positive control samples, negative control samples, buffers, cell culture media, etc.

Numerous aspects and advantages of the invention will be apparent to those skilled in the art upon consideration of the following detailed description which provides illumination of the practice of the invention.

EXAMPLES

The following examples are put forth so as to provide those of ordinary skill in the art with a complete disclosure and description of how to make and use the present invention, and are not intended to limit the scope of what the inventors regard as their invention nor are they intended to represent that the experiment bellow are all or the only experiments performed. Efforts have been made to ensure accuracy with respect to numbers used (e.g., amounts, temperature and etc.) but some experimental errors and deviations should be accounted for.

Example 1

Generating Recombinant Japanese Encephalitis Viral (JEV) Constructs

The JEV replicon J-R2A was constructed by steps as described below. CMV-RP-9-ribo-polyA/pBR322, a full-length infectious cDNA clone of Japanese encephalitis virus of Taiwan strain (RP-9, National Center for Biotechnology Information (NCBI) access No: AF014161; SEQ ID NO: 15) was prepared in accordance with the method described previously by Liang et al (Vaccine (2009) 27: 2746-2754). This cDNA clone was used to construct JEV replicon, J-R2A (FIG. **1**), in which structure genes of C-prM-E were removed and replaced by *Renilla* luciferase gene using jumping polymerase chain reaction (PCR). An FMDV-2A peptide sequence was then inserted behind the reporter gene so that *Renilla* luciferase may be precisely spliced with non-structure proteins after translation. Briefly, the C-prM-E gene of JEV cDNA clone was truncated using PCR. Primer prJE2388ApaKpnF (SEQ ID NO: 1,5'-gagggcccatggtacc atgggcgtcaacgcacga-3') and primer JE4507-4489 (−) (SEQ ID NO: 2,5'-cagaccttccatggaacac-3') was used to amplify JEV partial E to NS2 fragment; and the amplified product was placed into the C-prM-E portion of CMV-RP-9-ribo-polyA/pBR322 after ApaI and XmaI digestion. Then, *Renilla* luciferase gene was amplified using primers prJE181-197-AgeI-RLuc (SEQ ID NO: 3,5'-cataaactttcgaagtcataccggttac-tacc-ctcttcactc-3'), prJE1-25-F (SEQ ID NO: 4,5'-agaagtt-tatctgtgtgaacttctt-3') and prRLuc-NotKpnR (SEQ ID NO: 5,5'-ctggtaccggcggccgcttgttcatttttgagaactc-3') to restore partial Capsid for replication required CS sequence. After ApaI and KpnI digestion, the PCR product was inserted into the C-prM-E deleted cDNA clone. Finally, FMDV 2A peptide sequence was introduced into the construct by PCR amplification using primers prFMDV2A_F (SEQ ID NO: 6,5'-gctagcggccgccaacttcgacctcctcaagttggcgggagacg-3') and prFMDV2A_R (SEQ ID NO: 7,5'-ctggtacccggcccagggttg-gactcaacgtctcccgccaacttg-3'). After NotI and KpnI digestion, FMDV 2A sequence was inserted into above described cDNA clone and denoted as J-R2A (FIG. **1**).

J-R2A-M replicon was generated from J-R2A replicon by single-primer mutation, in which MfeI sites were silently removed with primers prJE_SP_NS2A__3856-3897(+) (SEQ ID NO: 8, 5'-tcctaggggctgccttttccagttagcctcagt-agatctgc-3') and prR2A_SP_polyA__10106-10147(+) (SEQ ID NO: 9, 5'-tgaacctgaaacataaaatgaatgcagttgttgttgttaacttgtt-3'). The

MfeI site at NS2A of J-R2A and MfeI site at delta ribozyme were both removed. The Infectious clone of RP9-XM was also constructed by replacing the corresponding region of J-R2A-M (FIG. 1), and the infectivity of virus recovered from RP9-XM was found to be similar to that from the parental RP9-X in mouse experiments.

Example 2

Characterization of the JEV NS1 Functionality by Use of NS1 C-terminal Truncated Mutants of J-R2A of Example 1

To study the relationship between JEV NS1 C-terminal amino acid and the NS1 functionality, several defective replicons J-R2A-dNS1 (i.e., NS1 C-terminal truncated mutants of J-R2A of Example 1) were generated and NS1 functionality of each defective replicon was analyzed by trans-complementation assays. Specifically, J-R2A-dNS1, and series of NS1 C-terminus truncated mutants including J-NS1.332/pCR3.1, J-NS1.337/pCR3.1, J-NS1.342/pCR3.1, and J-NS1.344/pCR3.1 were constructed. Each of the J-NS1 C-terminus truncated clones was subjected to trans-complementation assay, and 96 hrs after transfection, cell lysate of each clone was collected and replication ability of each clone was quantified by dual luciferase assay.

Briefly, 0.3 μg of each recombinant replicon DNA and 0.03 μg of pGL3 plasmid DNA, which served as the internal control, were mixed with 1.5 μl of Lipofectamine 2000 (Invitrogen, USA), and the mixture was used to transfect BHK-21 cells that were in 50-60% confluence in each well of the 24-well culture plate. For trans-complementation assay, 0.3 μg of recombinant J-NS1.flag/pCR3.1 plasmid DNA, 0.3 μg of replicon DNA, plus 0.03 μg of pGL3 plasmid DNA were mixed with Lipofectamine 2000 (Invitrogen, USA) and the mixture was used to co-transfect BHK-21 cells by steps as described above. 48 or 96 hrs after transfection, cell lysate of each clone was collected and luciferase activity of each clone was measured with commercial kits (Promega, Madison, Wis., USA) in accordance with the manufacturer's protocol. Results are depicted in FIG. 2.

J-R2A-dNS1 was a NS1 deletion mutant, in which 61-260 amino acids of NS1 were deleted, and it was found that such deletion resulted in the lost of viral replication capability (FIG. 2). On the other hand, if J-R2A-dNS1 was trans complemented with J-NS1.flag/pCR3.1, which expressed JEV NS1 protein by the CMV promoter, viral replication ability was restored (FIG. 2). Further, it was found that the C-terminal truncated mutant J-NS1.332/pCR3.1 failed in trans-complementation assay; and truncated mutants J-NS1.337/pCR3.1 showed reduced replication ability; whereas truncated mutants of J-NS1.342/pCR3.1 or J-NS1.344/pCR3.1 expressed complement replication ability in trans that was comparable to that of J-NS1.flag/pCR3.1.

Therefore, JEV NS1 (SEQ ID NO: 16), which is 352 amino acids in full length, requires at least 342 amino acids to render NS1 functional, shortening NS1 C-terminus may impair its replication ability. While very C-terminal 8 amino acids of NS1 is required for poly-protein processing to separate NS1 and NS2A (Falgout and Markoff, (1995) J Virol 69: 7232-7243), it is plausible that exogenous peptide may be inserted between 342 to 344 amino acid of the NS1 protein.

Example 3

Producing Fusion Protein Comprising JEV NS1 and Enterovirus 71 (EV71) SP70 Epitope

In this example, two restriction sites BssHII and PmlI were first introduced into NS1 C-terminus amino acids 343 and 344 by single-primer mutagenesis, and the construct was denoted as J-NS1.BP.flag/pCR3.1. Replication ability of such clone was slightly lower than that of J-NS1.flag/pCR3.1 in trans-complementation assay. Meanwhile, an exogenous peptide, Enterovirus 71 (EV71) SP70 epitope (YPTFGEHKQEKDLEY) (Foo et al., (2007) Virus Res 125: 61-68) was inserted into the above-described J-NS1.BP.flag/pCR3.1, and the resulted clone was denoted as J-NS1.EV71.flag/pCR3.1. The replication ability of the EV71 SP70 fused J-NS1.343SP70.flag/pCR3.1 clone was found to reduce to about 50% in trans-complementation assay (FIG. 3). This reduction in replication ability may be due to the lengthening of C-terminus of NS1 or the conformational changes of the NS1.

Further, J-R2A-343SP70 (FIG. 4) was constructed by common cloning techniques, including restriction enzyme digestion and ligation. In J-R2A-343SP70 clone, the EV71 SP70 was inserted between the JEV $NS1_{343}$ and the tail $polypeptide_{344}$. After verifying the sequence, plasmid DNA of J-R2A-343SP70 and J-R2A were transfected into the BHK-21 cells, the replication capabilities were quantified by dual luciferase assay system as described above. It was found that the J-R2A-343SP70 exhibited 50% replication activity of the parental J-R2A (FIG. 5), which indicates that the NS1 having an inserted EV71 SP70 epitope closed to its C-terminus has slightly effect on virus replication using replicon system.

Western blots were also performed to verify the expression of the fusion protein. Briefly, specific antiserum against JEV NS1 and EV71 VP1 respectively by J2-54 and PAB7630-B01P (Abnova Corp, USA) were used in this western blot assay. The transfected BHK-21 cells were treated with passive lysis buffer (Promega, Madison, Wis., USA) and total protein lysate was collected; 10% PAGE was then performed and transferred to PVDF membrane. Results are depicted in FIG. 6.

The anti J-NS1 antiserum exhibited specific binding to the monomer J-NS1, homo-dimer J-NS1 protein, as well as hetero-dimer of J-NS1 and J-NS1'. Results from FIG. 6 confirmed that NS1 may form dimers, and the dimerized NS1 has a molecular weight corresponds to that of EV71 SP70 epitope fused J-NS1, when compared with that of the authentic J-NS1 and protein size markers. The polyclonal PAB7630-B01P specifically recognized the dimeric J-NS1 proteins that fused with the EV71 SP70 epitope, instead of the authentic J-NS1 per se. These results demonstrate that the EV71 SP70 epitope may be fused with the JNS1 C-terminal, and subsequently be expressed in the live cells.

Furthermore, the infectious clone of JEV with EV71 SP70 fused at NS1 (RP9-SP70) was constructed, recombinant RP9-SP70 virus was generated, and then was used to infect BHK-21 cells. The culture medium of BHK-21 cells were collected and concentrated using PALL Nanosep device (MWCO=10K), so as to exam the secretion ability of NS1. After 10% PAGE separation and blotting to PVDF membrane, Western blots were performed with anti-JEV NS1 monoclonal antibody and anti-EV71 VP1 polyclonal antibody; the results are illustrated in FIG. 7.

It is confirmed that the recombinant JEV NS1-SP70 can be secreted into the culture medium just as the control virus RP-9, and the recombinant JEV NS1-SP70 also exists in dimeric form of the secreted NS1 fusion proteins.

Taken together, results from this example demonstrate that the recombinant JEV infectious clone, in which the NS1 C-terminus acts as a foreign peptide carrier, can be successfully expressed and secreted out of the infected cells.

Example 4

Producing Fusion Proteins Comprising JEV NS1 and Multiple Enterovirus 71 (EV71) SP70 Epitope

In this example, fusion proteins having 2, 3, and 4 copies of EV 71 SP70 epitopes, respectively, were produced according to similar procedures described in Example 3.

It is known that the J-R2A-343SP70 (1XSP70-J-R2A) construct contains a unique BssHII restriction site at 5' end of EV71-SP70 DNA sequence, and MluI and PmII restriction sites at the 3' end. While the BssHII and MluI are compatible restriction sites that can be ligated to each other, BssHII and PmII double digested EV71-SP70 PCR product as previously described were ligated to J-R2A-NS1-SP70, which was MluI and PmII digested. When BssHII and MluI are ligated, both restriction sites would be destroyed and thereby resulted in Ala-Leu di-amino acids between the EV71 SP70 epitopes. The resulted clone was therefore denoted 2XSP70-J-R2A. Meanwhile, 3XSP70-J-R2A and 4XSP70-J-R2A were also cloned. In this experiment, there are were 22, 39, 56 and 73 amino acids (SEQ ID NOs: 11, 12, 13, and 14, respectively) inserted between the JEV $NS1_{343}$ and the tail polypeptide$_{344}$ of 1XSP70-J-R2A, 2XSP70-J-R2A, 3XSP70-J-R2A and 4XSP70-J-R2A, respectively. The sequences of multiple EV71-SP70 clones were verified, and the plasmid DNA of multiple EV71-SP70 replicon clones were transfected into BHK-21 cells and subsequently verified by dual-luciferase assay. Results were depicted in FIG. **8**.

FIG. **8** illustrated that the replication activity of 2×SP70-J-R2A and 3×SP70-J-R2A was about 50% of that of J-R2A after 96-hours post transfection; whereas the replication activity of 4×SP70-J-R2A was less than 40% of that of J-R2A at 96-hour post transfection.

Example 5

Survival Plot of Animals Inoculated with 343SP70 Recombinant JEV Viruses

In this example, survival test of animals inoculated with the recombinant JEV virus were performed. Mice, which were intracerebrally (i.c.) injected with 10 micro-liter PBS to break down blood-brain barrier (BBB), were intraperitoneally (i.p.) injected with 2×10^5 pfu (plaque formation unit) of the RP9-XM, 2×10^5 pfu of the RP-2 ms and 2×10^5 pfu of the recombinant JEV virus having an inserted EV71 SP70 epitope (i.e., 343SP70), respectively. Animals inoculated with RP9-XM died after 10 days, whereas over 90% animals inoculated with the infectious clone RP-2 ms or the recombinant JEV virus (i.e., 343SP70) remained healthy 21 days after injection (FIG. **9**). The results demonstrate that the recombinant JEV virus of this invention is as attenuated as a vaccine strain RP-2 ms previously reported (Chen et al., Virology 223(1):79-88, 1996; Lin et al., Virus Res. 44(1):45-56, 1996; Liang et al., Vaccine 27(21):2746-2754, 2009).

Example 6

Inducing Specific Antibody Response in Mice Immunized with 343SP70

In this example, whether JEV 343SP70-RP9 could induce the immunized mice a specific antibody response to recognize SP70 epitope was investigated. First, sera were individually collected from the mice infected with JEV 343SP70. Then, a recombinant EV71 VP1 protein (P3386, Abnova) was used as an antigen and immunoblot assay was performed to examine the sera. As a positive control, polyclonal antibody PAB7630-B01P (Abnova) against EV71 VP1 was employed. The results illustrated in FIG. **10** suggest that neutralizing epitope SP70 fused with JEV NS1 could indeed elicit a specific antibody response from the immunized mice.

Example 7

Producing Fusion Protein Comprising JEV NS1 and *Gaussia* Luciferase

In this example, *Gaussia* luciferase (Gluc), a secreted protein with 185 amino acids in length was used to investigate the possible capacity for an insertion to be engineered into NS1 C-terminus.

First, a J-R2A-Gluc replicon was obtained by inserting Gluc (185 amino acids) between the JEV $NS1_{343}$ and the tail polypeptide$_{344}$. However, this replicon did not replicate as efficiently as its parental J-R2A replicon.

To solve this problem, an FMDV-2A peptide (NFDLLKLAGDVESNPGP, 17 amino acids) is fused in front of Gluc to obtain the J-R2A-2A-Gluc replicon. The J-R2A-2A-Gluc replicon, which comprises an exogenous polypeptide having 202 amino acids in length, appeared to replicate as well as the parental J-R2A replicon in the transfected BHK-21 cell by dual luciferase assay as described above (FIG. **11**).

Without being bound by theory, it is believed that the translational cleavage functionality of the FMDV-2A peptide may properly separate JEV NS1 from the Gluc thereby facilitating the replication process. This result suggests that the inclusion of a protease segment in the exogenous polypeptide may improve the insertion capacity of the recombinant viral construct.

Example 8

Producing Fusion Protein Comprising JEV NS1 and EV71 VP1 Epitope

EV71 capsid protein VP1 is consisted of 297 amino acid residues. Therefore, an approach similar to that used in EXAMPLE 6 was adopted to design a replicon carrying EV71 VP1 epitope. Specifically, the exogenous polypeptide consisting of the FMDV 2A peptide (17 amino acids), the secretion signal peptide (MGVKVLFALICIAVAEAGL, 19 amino acids) derived from Gluc and the partial EV71 VP1 epitope (corresponding to amino acid residues from 145^{th} to 261^{st}) were inserted between the JEV $NS1_{343}$ and the tail polypeptide$_{344}$ to produce a J-R2A-2A-Gss-VP1-C replicon. The total insertion size of this exogenous polypeptide was 153 amino acids in length. Dual luciferase assay indicated that J-R2A-2A-Gss-VP1-C replicon could replicate as efficiently as wild-type J-R2A replicon.

Example 9

Producing Fusion Proteins Comprising JEV NS1 and EV71 SP70 Epitope Inserted at Various Positions

In this example, various insertion positions were investigated to elucidate whether these positions are capable of accommodating the insertion of exogenous polypeptides without affecting infectivity of the recombinant JEV.

Specifically, as illustrated in FIG. **12**, six recombinant viral constructs $_{340341}$SP70, $_{340344}$SP70, $_{343341}$SP70, $_{343344}$SP70,

$_{352341}$SP70, and $_{352344}$SP70 were constructed. These constructs were denoted in accordance with the amino acid sequences immediately before and after the EV71 SP70 antigen being inserted into the JEV NS1. For example, the cDNA clone of $_{340341}$SP70 denotes that the EV71 SP70 antigen was inserted between the JEV NS1$_{340}$ and the tail polypeptide$_{341}$ of the JEV NS1, whereas the cDNA clone of $_{352344}$SP70 denotes that the EV71 SP70 antigen was inserted between the JEV NS1$_{352}$ and the tail polypeptide$_{344}$.

Results from the constructed recombinant JEV vectors clearly indicate that the region surrounding the 343th amino acid of JEV NS1 (e.g., the region including amino acids 340 to 352) is flexible for insertion of heterologous gene segment without significantly compromising the infectivity of the resultant recombinant JEV.

The expression of NS1-SP70 fusion proteins were analyzed by Western blot using NS1 specific antibody or SP70 specific antiserum and the results are summarized in FIG. **13**. Specifically, after 48-hour infection, culture medium was collected and concentrated as described above. Then, Western blot was performed using anti-serum J2-54 for JEV NS1 or anti-serum PAB7630-B01P (Abnova) for EV71 VP1. All the recombinant JEV tested could cause the infected cells to release NS1-SP70 fusion proteins into cultured medium, and more importantly, these fusion proteins could properly form dimer as the wild-type NS1 protein derived from RP-9 infection did. These results demonstrated JEV NS1 C-terminus is suitable for foreign peptide fusion and the fusion does not seem to interfere with the major functions of NS1 for JEV replication.

Example 10

Survival Plot of Animals Inoculated with $_{343341}$SP70 and $_{352341}$SP70 Recombinant JEV Viruses

In this example, survival test of animals inoculated respectively with the recombinant $_{343341}$SP70 and $_{352341}$SP70 JEV viruses were investigated in accordance with similar procedures described above in Example 5.

Animals inoculated with RP9-XM died within 10 days. By contrast, for animals inoculated respectively with the $_{343341}$SP70 and $_{352341}$SP70 recombinant JEV viruses, over 75% and 55% animals remained healthy 21 days after injection (FIG. **14**). The results demonstrate that the recombinant JEV virus of this invention exhibited attenuated phenotype to the mice tested when compared with wild-type RP-9.

Example 11

Inducing Specific Antibody Response in Immunized Mice

In this example, whether JEV $_{352341}$SP70 could induce the immunized mice a specific antibody response to recognize SP70 epitope was investigated in accordance with similar procedures described above in Example 6. The results illustrated in FIG. **15** suggest that the mice immunized with $_{352341}$SP70 could induce strong antibody response to EV71 VP1.

It will be understood that the above description of embodiments is given by way of example only and that various modifications may be made by those with ordinary skill in the art. The above specification, examples and data provide a complete description of the structure and use of exemplary embodiments of the invention. Although various embodiments of the invention have been described above with a certain degree of particularity, or with reference to one or more individual embodiments, those with ordinary skill in the art could make numerous alterations to the disclosed embodiments without departing from the spirit or scope of this invention.

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<210> SEQ ID NO 16
<211> LENGTH: 352
<212> TYPE: PRT
<213> ORGANISM: Japanese encephalitis virus
<220> FEATURE:
<223> OTHER INFORMATION: NS1

<400> SEQUENCE: 16

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Lys Tyr Leu Pro Glu Thr Pro Arg Ser Leu Ala Lys Ile Val His Lys
        35                  40                  45

Ala His Lys Glu Gly Val Cys Gly Val Arg Ser Val Thr Arg Leu Glu
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His Gln Met Trp Glu Ala Val Arg Asp Glu Leu Asn Val Leu Leu Lys
65                  70                  75                  80

Glu Asn Ala Val Asp Leu Ser Val Val Val Asn Lys Pro Val Gly Arg
                85                  90                  95

Tyr Arg Ser Ala Pro Lys Arg Leu Ser Met Thr Gln Glu Lys Phe Glu
            100                 105                 110

Met Gly Trp Lys Ala Trp Gly Lys Ser Ile Leu Phe Ala Pro Glu Leu
        115                 120                 125

Ala Asn Ser Thr Phe Val Val Asp Gly Pro Glu Thr Lys Glu Cys Pro
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Asp Glu His Arg Ala Trp Asn Ser Met Gln Ile Glu Asp Phe Gly Phe
145                 150                 155                 160

Gly Ile Thr Ser Thr Arg Val Trp Leu Lys Ile Arg Glu Glu Ser Thr
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Asp Glu Cys Asp Gly Ala Ile Ile Gly Thr Ala Val Lys Gly His Val
            180                 185                 190

Ala Val His Ser Asp Leu Ser Tyr Trp Ile Glu Ser Arg Tyr Asn Asp
        195                 200                 205

Thr Trp Lys Leu Glu Arg Ala Val Phe Gly Glu Val Lys Ser Cys Thr
    210                 215                 220

Trp Pro Glu Thr His Thr Leu Trp Gly Asp Gly Val Glu Glu Ser Glu
225                 230                 235                 240

Leu Ile Ile Pro His Thr Ile Ala Gly Pro Lys Ser Lys His Asn Arg
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Arg Glu Gly Tyr Lys Thr Gln Asn Gln Gly Pro Trp Asp Glu Asn Gly
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Ile Val Leu Asp Phe Asp Tyr Cys Pro Gly Thr Lys Val Thr Ile Thr
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Glu Asp Cys Gly Lys Arg Gly Pro Ser Val Arg Thr Thr Thr Asp Ser
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Gly Lys Leu Ile Thr Asp Trp Cys Cys Arg Ser Cys Ser Leu Pro Pro
305                 310                 315                 320

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Pro Val Arg His Asp Glu Thr Thr Leu Val Arg Ser Gln Val Asp Ala
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What is claimed is:

1. An isolated recombinant viral construct for expressing an exogenous polypeptide in a cell, comprising:

a Japanese encephalitis virus (JEV) replicon comprising a nucleic acid encoding a fusion protein comprising in sequence:

a JEV non-structural protein 1 (JEV NS1) segment comprising at least amino acid residues 1 to 340 of SEQ ID NO: 16;

the exogenous polypeptide having at least 6 amino acids; and

a tail polypeptide comprising at least amino acid residues 344 to 352 of SEQ ID NO: 16, wherein the production of the fusion protein does not affect viral replication.

2. The isolated recombinant viral construct of claim **1**, wherein the exogenous polypeptide comprises an immunogenic segment.

3. The isolated recombinant viral construct of claim **2**, wherein the immunogenic segment is an Enterovirus 71 (EV71) SP70 antigen, an EV71 VP1 antigen or a portion thereof.

4. The isolated recombinant viral construct of claim **1**, wherein the tail polypeptide comprises amino acid residues 341 to 352 of SEQ ID NO: 16.

5. The isolated recombinant viral construct of claim **1**, wherein the JEV NS1 segment is amino acid residues 1 to 343 of SEQ ID NO: 16, and the tail polypeptide is amino acid residues 341 to 352 of SEQ ID NO: 16.

6. The isolated recombinant viral construct of claim **1**, wherein the JEV NS1 segment is amino acid residues 1 to 343 of SEQ ID NO: 16, and the tail polypeptide is amino acid residues 344 to 352 of SEQ ID NO 16.

7. The isolated recombinant viral construct of claim **1**, wherein the JEV NS1 segment is amino acid residues 1 to 352 of SEQ ID NO: 16, and the tail polypeptide is amino acid residues 341 to 352 of SEQ ID NO: 16.

8. The isolated recombinant viral construct of claim **1**, wherein the JEV NS1 segment is amino acid residues 1 to 352 of SEQ ID NO: 16, and the tail polypeptide is amino acid residues 344 to 352 of SEQ ID NO: 16.

9. The isolated recombinant viral construct of claim **2**, wherein the exogenous polypeptide further comprises a protease segment preceding the immunogenic segment, wherein the protease segment comprises a Foot-and-Mouth Disease virus 2A (FMDV-2A) peptide, and the exogenous polypeptide is at least 100 amino acids in length.

10. The isolated recombinant viral construct of claim **9**, wherein the exogenous polypeptide further comprises a secretion-signal segment between the protease segment and the immunogenic segment, wherein the secretion-signal segment comprises a secretion signal peptide derived from *Gaussia* luciferase.

11. The isolated recombinant viral construct of claim **1**, wherein the JEV replicon comprises a CMV promoter operably linked thereto such that the cell may express and subsequently secrete the fusion protein out of the cell.

12. The isolated recombinant viral construct of claim **1**, wherein the cell is a BHK-21 cell, a C6/36 cell, or a Vero cell.

13. An isolated recombinant JEV particle, comprising a virion unit comprising an isolated recombinant viral construct of claim **1**.

14. The isolated recombinant JEV particle of claim **13**, wherein the exogenous polypeptide comprises an immunogenic segment, wherein the immunogenic segment is an Enterovirus 71 (EV71) SP70 antigen, an EV71 VP1 antigen or a portion thereof.

15. The isolated recombinant JEV particle of claim **13**, wherein the exogenous polypeptide comprises an immunogenic segment and a protease segment preceding the immunogenic segment, wherein the protease segment comprises a Foot-and-Mouth Disease virus 2A (FMDV-2A) peptide, and the exogenous polypeptide is at least 100 amino acids in length.

16. A method of eliciting an immune response in a host, comprising administering the isolated recombinant JEV particle of claim **13** to the host, wherein said administering provides for expression of the exogenous polypeptide that results in induction of an immune response in the host to the exogenous polypeptide.

17. The method of claim **16**, wherein the exogenous polypeptide comprises an immunogenic segment, wherein the immunogenic segment is an Enterovirus 71 (EV71) SP70 antigen, an EV71 VP1 antigen or a portion thereof.

18. The method of claim **16**, wherein the exogenous polypeptide comprises an immunogenic segment and a protease segment preceding the immunogenic segment, wherein the protease segment comprises a Foot-and-Mouth Disease virus 2A (FMDV-2A) peptide, and the exogenous polypeptide is at least 100 amino acids in length.

19. The isolated recombinant JEV particle of claim **15**, wherein the exogenous polypeptide further comprises a secretion-signal segment between the protease segment and the immunogenic segment, wherein the secretion-signal segment comprises a secretion signal peptide derived from *Gaussia* luciferase.

20. The method of claim **18**, wherein the exogenous polypeptide further comprises a secretion-signal segment between the protease segment and the immunogenic segment, wherein the secretion-signal segment comprises a secretion signal peptide derived from *Gaussia* luciferase.

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